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DEFINING ORAL MICROBIOLOGICAL HEALTH

James Oliver Kistler

A thesis submitted in accordance with the requirements of the degree of Doctor of Philosophy in King's College London

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Abstract

Background: The composition of the oral microbiota in periodontal health and the microbial changes associated with the early stages of gingivitis are poorly defined.

Aims: The aims of this work were to: (i) characterise the bacterial composition of dental plaque in subjects with experimentally-induced gingivitis; (ii) identify candidate oral probiotic taxa; (iii) evaluate alternative approaches for oral *Neisseria* spp. differentiation.

Methods: Twenty volunteers abstained from oral hygiene in the mandible for two weeks. Clinical indicators of inflammation were monitored and samples of plaque were analysed, together with 20 control samples from periodontitis patients, by pyrosequencing of 16S rRNA genes and culture. A panel of oral bacterial isolates were screened for inhibition of six indicator organisms using a deferred antagonism assay. Sequencing of seven 'housekeeping' genes and a ribosomal protein gene (*rplF*) were evaluated as methods to differentiate *Neisseria* species.

Results: All volunteers developed gingivitis after two weeks. 344,267 16S rDNA sequences were clustered into a median of 299 species-level Operational Taxonomic Units (OTUs) per sample. Principal Coordinate Analysis plots revealed shifts in community structure with gingivitis development, and the mean Simpson's inverse diversity index increased from 32 at baseline to 47.5 after two weeks (P<0.0001). *Fusobacterium nucleatum* subsp. *polymorphum*, *Lautropia* sp. HOTA94, *Lachnospiraceae* sp. HOT100 and *Prevotella oulorum* were significantly associated with gingivitis, whilst *Rothia dentocariosa* was health-associated. Of 80 isolates screened, two strains of *Streptococcus cristatus* and a *Streptococcus* sp. HOT071 strain, inhibited the growth of one or more of *Streptococcus anginosus*,

Solobacterium moorei, *Porphyromonas gingivalis* and *Filifactor alocis*. *rplF* gene sequences were found to reliably differentiate oral *Neisseria* species, although some taxonomic revision to the genus is indicated.

Conclusions: A highly species-rich bacterial community in health-associated plaque was revealed and new health- and gingivitis-associated taxa were identified. Three strains were found with potential for use as oral probiotics.

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Chapter 1:
Introduction

Chapter 1: Introduction

The oral cavity is one of the principal portals of entry to the human body and is involved in a number of important processes including communication, sensory activities and the initial stages of digestion. It is also host to a highly diverse array of microorganisms, known collectively as the oral microbiome (Dewhirst et al, 2010). These microorganisms play a major role in the aetiology of common oral diseases including dental caries (tooth decay) and the periodontal diseases: gingivitis (inflammation of the gingivae) and chronic periodontitis (inflammation and destruction of the periodontium). Dental caries and periodontal disease are highly prevalent and constitute a substantial global public health burden. Data from the World Health Organization's global Oral Health Report shows that severe periodontitis, for example, affects 5-15% of most adult populations in the 35-44 year old age range (Petersen, 2004). More recently, based on a sample of the United States population, it was estimated that 47.2% of American adults aged 30 years or older have mild, moderate or severe periodontitis (Eke et al., 2012). As well as causing pain, discomfort and adversely affecting normal functions within the oral cavity itself, there is increasing evidence that oral diseases can impact general health and wellbeing. Periodontitis, for instance, has been linked to coronary heart disease (Humphrey et al., 2008) and diabetes mellitus (Lalla and Papapanou, 2011). Tooth loss, a surrogate marker of periodontal disease, was also recently associated with an increased risk of dementia and cognitive decline (Batty et al., 2013). In addition, poor oral hygiene and gingival bleeding after tooth brushing have been identified as risk factors for bacteraemia which can lead to infections at other body sites (Lockhart et al. 2009). For example, oral bacteria have been identified as the causative agents

of a case of intra-uterine infection leading to a premature birth (Han et al., 2006b) and infective endocarditis (Knox and Hunter, 1991).

Decades of research have led to the current hypothesis that dental caries and periodontal diseases are the result of environmental perturbations that lead to a breakdown of host-microbiome homeostasis in the oral cavity and enable, or favour, the proliferation of putatively pathogenic, or disease-associated, species (Marsh, 2003, Darveau, 2010). Whilst specific caries- and periodontitis-associated bacteria have been identified, the bacterial species that are important in the initial stages of these diseases remain largely unknown. Furthermore, a comprehensive knowledge of the composition of the oral microbiome in health has yet to be obtained, thereby hindering attempts to elucidate the precise changes that lead to disease.

1.1 The oral cavity and its resident microbiome

The oral cavity is a relatively open environment that, under normal conditions, is sufficiently moist, rich in nutrients, and of a temperature (34-36°C) and pH (6.75-7.25 in saliva) that is ideal for the growth of a range of different microorganisms attached to intra-oral surfaces as biofilms (Marcotte and Lavoie, 1998, Marsh et al., 2011). The successful colonisation of these surfaces, however, relies upon a microorganism's ability to overcome or tolerate numerous environmental challenges such as physical removal by salivary fluid flow (Rosan and Lamont, 2000) and the host immune response (Cole et al., 1999). These selective pressures and differences in the prevailing environmental conditions result in the development of a microbial community, or microbiome, that is different to that found at other human body sites such as the colon and on the skin (Moore and Moore, 1994, Bik et al., 2010, Fierer et al., 2010). Within the oral cavity itself there are a number of different components and surfaces that constitute ecologically distinct microbial habitats or ecological

niches (Socransky and Haffajee, 2005). These include the non-shedding surfaces of the teeth, the hardest tissue in the human body, and the mucosal shedding soft surfaces of the tongue, cheeks, lips and palate. Physical properties including pH, redox potential and temperature, among others, vary across intra-oral sites resulting in considerable intra-oral variability in the composition of the oral microbiome (Aas et al., 2005, Segata et al., 2012). Furthermore, at a given site, such properties may be subject to temporal changes. For example, induction of an inflammatory immune response in the gingival crevice may raise the local temperature (Fedi and Killoy, 1992), favouring the growth of particular species and resulting in a shift in the composition of the bacterial community present (Marsh and Devine, 2011).

By definition, the oral microbiome includes all the microorganisms that are found on or in the human oral cavity (Dewhirst et al., 2010). Whilst *Archaea*, fungi, protozoa and viruses are all found in the oral cavity, it is bacteria that are the most numerous (Do et al., 2013) and will form the focus of this investigation. Henceforth, the term 'oral microbiome' will refer solely to the bacterial domain. The Human Oral Microbiome Database (HOMD), a curated database of reference 16S rRNA gene sequences for oral bacteria, currently (reference set version 13.2) consists of over 800 different species from 13 different bacterial phyla: *Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Firmicutes, Fusobacteria,* GN02, *Proteobacteria, Spirochaetes,* SR1, *Synergistetes, Tenericutes* and TM7 (Dewhirst et al., 2010). The majority of these species are members of the six phyla *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria,* and *Spirochaetes,* with the phylum *Firmicutes* containing the greatest number. Interestingly, only 65.6% of these taxa have been cultivated *in vitro* (Dewhirst et al., 2010). Indeed, the entire un-named Divisions TM7 and SR1 remain without

cultivable representatives, whilst the majority of oral *Synergistetes* and *Spirochaetes* phylotypes are yet to be cultured. The uncultivated un-named Division: GN02, has been detected in the human oral cavity recently using deep sequencing methods and is reportedly also a member of the canine oral microbiome (Dewhirst et al., 2012). Among those oral taxa that have been cultured and described, a wide range of both Gram -negative, and -positive, aerobic and facultatively or obligately anaerobic, cocci, rods, spirochaetes and filaments are represented. These taxa were identified in a range of different samples taken from tooth and mucosal surfaces, both in health and in different disease states, and therefore may include species that are not ordinarily present under normal conditions in healthy individuals. There are data to suggest that a particular individual is likely to be colonised by only a subset of these oral taxa at a given time. For example, one deep sequencing study of the oral microbiome in three healthy individuals detected a total of 818 species-level phylotypes (Keijser et al., 2008). On average 266 species-level phylotypes were found per oral cavity (sampled at different intra-oral sites) and 313 phylotypes were shared between the three microbiomes. Transient exogenous taxa are also frequently found in the oral cavity (Dewhirst et al., 2010). Transient taxa likely lack the appropriate adhesion molecules necessary for successful colonisation of oral surfaces, or biofilms, and therefore do not persist in the oral environment (Nobbs et al., 2011).

As a result of our inability to culture many oral bacteria, a combination of both culture-independent molecular methods, such as 16S ribosomal RNA (rRNA) gene cloning and sequencing, and traditional cultural methods, have been required in order to gain the current knowledge of the composition of the oral microbiome. Studies employing these approaches have revealed that oral bacterial communities

are variable in terms of their membership and structure, both intra-orally and inter-individually (Mager et al., 2003, Aas et al., 2005, Bik et al., 2010, Segata et al., 2012).

Intra-orally, as already noted, a variety of different habitats exist which can favour the growth of different consortia of bacterial species. Furthermore, communities at specific sites within the oral cavity of a given individual display a degree of temporal variation, particularly with changing health status of the site (Kumar et al., 2006). The hard non-shedding surfaces of the teeth provide an environment that is especially suited to the growth of communities of bacteria in the form of biofilms known as dental plaque (Marsh, 2004). The lack of desquamation of the tooth surface enables plaque to accumulate relatively undisturbed, unless effective oral hygiene is practised, resulting in a thick biofilm with a high bacterial load (Loesche and Syed, 1978). Different sites on the teeth can support the growth of plaque communities with varying composition. For example, subgingival plaque forming on the portion of a tooth within the gingival crevice can differ in composition to supragingival plaque forming above the gingival margin on the smooth surface of the same tooth (Socransky and Haffajee, 2005). The gingival crevice provides a distinct environment to that of the tooth above the gingival margin, primarily due to the influx of gingival crevicular fluid, which is composed of host-derived proteins that can be a source of nutrients for proteolytic anaerobic bacteria such as *Prevotella* spp. and *Porphyromonas* spp. (Marsh, 2003). Mucosal surfaces such as the lips, cheek, palate and tongue, have been found to harbour distinct bacterial communities to those found on tooth surfaces, due to continual shedding and likely different surface receptors for bacterial adhesion (Mager et al., 2003, Aas et al., 2005). A recent study characterising the human digestive tract

microbiome in health, clustered oral sites into separate groups according to similarities in their bacterial community structure (Segata et al., 2012). The buccal mucosa, keratinized gingiva and hard palate formed the first group, the saliva, tongue, tonsils, and throat the second, and supragingival and subgingival plaque the third.

A study analysing the composition of the oral microbiota in 10 healthy individuals revealed substantial inter-individual variation with respect to the presence/absence and proportions of the major bacterial genera and species from different parts of the mouth (Bik et al., 2010). Similarly, using a retrievable enamel chip model Diaz et al. (2006) found marked inter-individual differences in the composition of bacterial communities shortly after the initial colonisation of the enamel. In particular, both the prevalence and relative proportions of the class *Clostridia* and the genera *Abiotrophia*, *Corynebacterium*, *Neisseria*, *Rothia* and *Prevotella* were highly variable between individuals. This variability may be attributed to host factors that can affect the colonisation and growth of particular bacteria, including the host's immune system and genetics (Marsh and Devine, 2011). In addition, lifestyle factors, such as an individual's diet, may impact the growth and resulting proportions of particular species in dental plaque (Bowden and Li, 1997). One excellent example of this is the frequent consumption of fermentable carbohydrates, subsequent bacterial metabolism of which results in the production of acids that lower the pH of plaque thereby favouring the growth of acidogenic and aciduric bacteria such as mutans-streptococci and lactobacilli which are associated with dental caries (Bradshaw et al., 1989). However, it is thought that other dietary components have less of a significant impact on the composition of the microbiome (Wade, 2013). One notable lifestyle factor affecting the oral microbiome is cigarette

smoking. One study that investigated the effect of smoking found that the microbial profiles in periodontitis patients who smoked were different to those in patients who had never smoked, with the species: *Eubacterium nodatum*, *Fusobacterium nucleatum* subsp. *vincentii*, *Prevotella intermedia*, *Peptostreptococcus micros* (now *Parvimonas micra*) *Prevotella nigrescens*, *Bacteroides forsythus* (now *Tannerella forsythia*), *Porphyromonas gingivalis* and *Treponema denticola* being more prevalent among current smokers (Haffajee and Socransky, 2001, Shchipkova et al., 2010). The impact of host genetics on the oral microbiome was examined in a recent longitudinal study of the salivary microbiota of twins from early adolescence to early adulthood, and showed that given twins harboured a more similar microbiota at all time points, when compared to the whole population analysed (Stahringer et al., 2012). They also showed that this similarity declined with age, and in particular when they no longer co-habited, suggesting that the shared environment was also an important determinant of the species present. The analyses did not, however, detect a significant impact of weight, gender or food preferences on the composition of the salivary microbiota.

Despite the extensive inter-individual variability of the oral microbiome reported by the studies described above, distinct and characteristic oral bacterial community configurations have been associated with oral health and different dental diseases. For example, the subgingival plaque communities of individuals with severe chronic periodontitis are characterised by having greater proportions of obligately anaerobic and proteolytic Gram-negative species including *Prevotella* spp., *Synergistetes* and *Spirochaetes,* as well as particular Gram-positive anaerobes such as *Eubacterium spp*., relative to healthy individuals (Griffen et al., 2012, Abusleme et al., 2013). Moreover, a number of species have been strongly associated

with the disease and the presence of each other, such as the so-called 'red complex' (*P. gingivalis*, *T. forsythia* and *T. denticola*) (Socransky et al., 1998), which will be discussed in greater detail in a later section.

1.2 Dental plaque

Dental plaque is a biofilm composed of polymicrobial communities structurally organised within a matrix of extracellular polymers (Marsh, 1994). Because plaque aggregates on the teeth and around the gingival margin it plays a fundamental role in both the aetiology of dental caries and periodontal diseases. The development of plaque is reported to occur in a structured and sequential manner and is characterised by a transition in bacterial community composition with age. Studies have shown that the initial plaque community is dominated by Gram-positive aerobic and facultative anaerobes and as it matures it becomes increasingly complex with greater numbers of Gram-negative and anaerobic species (Theilade et al., 1966, Moore et al., 1982, Rosan and Lamont, 2000). Using microscopy to examine plaque samples from healthy individuals removed at different time points during the absence of oral hygiene, Theilade et al. (1966) concluded that the bacteriological formation of plaque had occurred in three phases. The first phase occurred within the first two days, and involved the proliferation of Gram-positive cocci and rods, with Gram-negative cocci and rods appearing. In the second phase (1-4 days) fusobacteria and filamentous bacteria began to appear and increase, and in the third phase (4-9 days) spirilla and spirochetes also appeared.

Following cleaning or eruption of a tooth, a thin film of host-derived molecules, known as the acquired salivary pellicle, forms on the tooth surface (Whittaker et al., 1996). The salivary pellicle is composed of a number of proteins and glycoproteins such as the mucins: MUC5B and MUC7, and salivary α-amylase

(Al-Hashimi and Levine, 1989). The development of dental plaque begins with the adherence of the initial, or primary, colonising species to specific receptors in the salivary pellicle (Socransky and Haffajee, 2005, Marsh et al., 2011). Both culture-based and culture-independent studies characterising early plaque have found that the primary colonisers are predominantly species of the genera *Streptococcus* and *Actinomyces* (Loe et al., 1965, Theilade et al., 1966, Ritz, 1967, Moore et al., 1987, Diaz et al., 2006). Using a targeted molecular DNA-DNA hybridisation technique, Li et al. (2004) attempted to identify the early bacterial colonisers of dental plaque forming on the teeth of healthy volunteers shortly after cleaning. The most abundant colonisers in the earliest stage of plaque formation (<2hours) were *Actinomyces* spp., whilst the relative proportions of streptococci, particularly *Streptococcus mitis* and *Streptococcus oralis,* increased with time to become the dominant species after six hours. *Streptococcus* spp. were also the predominant species in a cultural investigation of the bacterial composition of four-hour dental plaque samples from caries-active and caries-inactive individuals (Nyvad and Kilian, 1990). The species most frequently detected were *Streptococcus sanguinis*, *S. oralis* and *S. mitis* biovar 1. More recently, Diaz et al. (2006) used an open-ended molecular method to characterise the microbial diversity of early dental plaque in three subjects, and also found that streptococci dominated the four- and eight-hour bacterial communities. However, the authors detected a significantly greater bacterial diversity present in early plaque than culture studies had suggested, with representatives of the genera *Gemella, Granulicatella, Neisseria, Prevotella, Rothia,* and *Veillonella* all frequently present in significant proportions. The ability of a number of the primary colonisers, identified in such studies, to adhere to specific receptors of the acquired salivary pellicle via adhesion molecules has been demonstrated. *S. sanguinis*, *Streptococcus*

gordonii and *S. oralis* were shown to be able to adhere to salivary α-amylase in addition to other receptors (Murray et al., 1992), whilst Gibbons et al. (1988) showed that *Actinomyces viscosus* (now *Actinomyces oris*) type 1 fimbriae mediate binding to salivary proline-rich protein 1 and statherin.

Once the primary colonisers have attached to the tooth surface via the pellicle and begin to multiply, secondary, or late, colonisers including other *Actinomyces* spp., *Haemophilus* spp., *Fusobacterium* spp., *Prevotella* spp., and *Veillonella* spp., adhere to the developing biofilm (Diaz et al., 2006, Kolenbrander et al., 2006). Primary and secondary colonisers of plaque can adhere to one another via the process of co-aggregation, which involves specific cell-to-cell recognition between genetically distinct cell types (Gibbons and Nygaard, 1970, Kolenbrander, 2000). This cell-cell recognition is mediated by adhesins and receptors on the bacterial cell surfaces and can occur between a number of different species (Kolenbrander et al., 2006). The specific co-aggregation interactions between species of streptococci and *Actinomyces*, for example, are well documented and extensive testing has de-lineated six 'co-aggregation groups' of each genus based on their ability to co-aggregate with one another (Kolenbrander and Andersen, 1990, Kolenbrander et al., 2006). Inter-generic co-aggregation can result in some of the interesting structures observed in dental plaque by Scanning Electron Microscopy (SEM), such as the 'corn-cob' formations that consist of coccal cells attached to a central filamentous cell (Saxton, 1973, Listgarten et al., 1975). Interestingly, a recent study examining plaque architecture using Fluorescent in-situ hybridisation (FISH) revealed 'corn-cob' formations that were the result of co-aggregation between *Streptococcus* spp. and the yeast *Candida albicans* (Zijnge et al., 2010). Co-aggregation testing has shown that, of the organisms tested, fusobacteria are able to co-aggregate with the greatest

number of different genera, including other later colonisers such as *Selenomonas* spp. and *Eubacterium* spp. (Kolenbrander et al., 1989, George and Falkler, 1992). As such fusobacteria have been purported to act as important intermediate bridging organisms between the early and late colonisers of plaque (Kolenbrander et al., 2006). In addition, *F. nucleatum* subsp. *polymorphum* has been shown to co-aggregate with *T. forsythia* (Sharma et al., 2005b) and *F. nucleatum* subsp. *nucleatum* with *P. gingivalis* (Kolenbrander et al., 2006), both of which are members of the 'red complex' putative periodontal pathogens (Socransky et al., 1998). This may suggest an important role for such bridging organisms in the successful colonisation of putative periodontal pathogens and thus in the initiation or progression of periodontal disease.

As dental plaque accumulates and its complexity increases, environmental conditions such as redox potential and pH within the developing biofilm may also change, potentially favouring the growth of organisms that are unable to grow in the initial biofilm. For example, oxygen consumption by early colonising aerobes and facultative anaerobes such as *Neisseria* spp. can lower the redox potential within developing plaque facilitating the growth of obligate anaerobes such as *Prevotella* spp. (Bradshaw et al., 1996). Indeed, it has been suggested that obligately anaerobic putative periodontal pathogens may survive in early developing plaque through close associations with organisms that are able to metabolise oxygen (Kolenbrander et al., 2006). Enabling survival of community members to oxygen exposure is just one of the means by which bacteria within plaque can co-operate. The establishment of nutritional networks is another such example. Metabolism of certain nutrients such as salivary carbohydrates by primary colonisers can result in metabolic end products that can subsequently be used as a nutrient source by other colonisers (Marsh, 2006).

One well-documented example is the production of lactic acid through fermentation of sugars by streptococci, which can then be used as a primary nutrient source for *Veillonella* spp. (Mikx and van der Hoeven, 1975). A form of communication known as quorum sensing enables bacteria to estimate the number of cells in their immediate surroundings via diffusible signalling molecules or 'autoinducers', in order to regulate their growth within the biofilm (Williams, 2007). Threshold concentrations of a particular autoinducer can induce the expression or repression of target genes that may be important in growth or pathogenesis. A number of quorum sensing pathways have been described in both Gram-positive and Gram-negative bacteria, the first of which was the N-acyl homoserine lactone induced production of light in the marine organism *Vibrio fisheri* (Nealson and Hastings, 1979). Evidence of a similar quorum sensing pathway that uses a different signalling molecule, designated Autoinducer 2 (AI-2), has been found in a number of oral bacteria, including fusobacteria, *P. gingivalis*, *P. intermedia* and *S. gordonii* (Shao and Demuth, 2010). Autoinducer 2 type quorum sensing can occur between different species (Federle, 2009), and it was shown to be essential for mixed species biofilm formation of *S. gordonii* and *P. gingivalis* (McNab et al., 2003). One recent in-vitro study demonstrated that the production of AI-2 by *F. nucleatum* subsp. *nucleatum* enhanced both the growth of mono- and dual- species biofilms (*F. nucleatum* with each of the 'red complex' species) and coaggregation (Jang et al., 2013). In addition, the authors revealed that *F. nucleatum* susbp. *nucleatum* AI-2 was able to induce the expression of surface molecules important in bacterial adhesion for each of the species tested.

1.3 Methods for the characterisation and analysis of the oral microbiome

1.3.1 *Characterisation of human-associated microbial communities*

There is presently an increased recognition among microbiologists and health professionals of the importance in gaining knowledge of the complex resident microbial communities, or microbiomes, colonising different sites on, or in, the human body. The multi-centre collaborative Human Microbiome Project (Peterson et al., 2009) aims to improve the understanding of human health and disease by comprehensively characterising these microbial communities, specifically whether changes in human microbiome composition or function can be related to the onset of particular diseases.

Microbiological studies of infectious diseases in the 19th and 20th centuries were largely focused on isolating specific pathogenic species in pure culture from diseased sites in order to establish causation based on Koch's postulates (Fredericks and Relman, 1996). This approach was successful in determining the bacterial causes of many important infectious diseases such as tuberculosis. In practice, however, this approach is inadequate for the study of complex multi-factorial diseases with a polymicrobial aetiology and/or diseases at sites with a diverse resident microbiome (Jenkinson and Lamont, 2005). This includes common diseases of the oral cavity such as chronic periodontitis, which is caused by the complex interactions between the oral microbiome and the host immune response (Kornman, 2008). In such diseases, knowledge of the bacterial communities present at the relevant site and an understanding of their interactions with the host, are important in elucidating the underlying causes. Increased knowledge of microbial communities and plaque biofilms in the human oral cavity has led to the establishment of an 'ecological plaque hypothesis' to explain the relationship between these communities and the

host, both in health and disease (Marsh, 2006). When applied to chronic periodontitis, this hypothesis can be summarised as a selection for, and increased proportions of, putative periodontal pathogens or periodontitis-associated species in dental plaque at the expense of health-associated species, due to changes in the local environment e.g. inflammation resulting in increased gingival crevicular fluid flow. It has been suggested that the health-associated members of the oral microbiome, when absent from a particular site, may be as important in the initiation and progression of disease as the putative pathogens themselves (Socransky and Haffajee, 2005). A recently emergent hypothesis known as the 'keystone pathogen' hypothesis postulates that microbial pathogens present at low relative abundances in the microbiome can induce inflammatory diseases, such as chronic periodontitis, by inducing a shift in the commensal microbiome from a benign (or health-associated) to dysbiotic state (Hajishengallis et al., 2012). There has therefore been an increased interest in defining the composition of the oral microbiome associated with health and identifying specific health-associated bacterial species (Tanner et al., 1998, Kazor et al., 2003, Aas et al., 2005, Jenkinson and Lamont, 2005, Keijser et al., 2008, Lazarevic et al., 2009, Zaura et al., 2009, Bik et al., 2010). Obtaining this knowledge will help our understanding of the perturbations that can lead to disease and may be used to inform the development of new prevention and treatment strategies.

As a result of the increasing emphasis placed on characterising complex human-associated bacterial communities and of technological advancements, the methods employed to detect and enumerate bacteria in the oral cavity have changed considerably over the course of recent decades. Early studies of oral bacterial communities were heavily reliant on the culture of bacteria *in vitro* using artificial

media and on light and electron microscopy. Sensitive molecular culture-independent approaches have become available in recent years and are becoming increasingly high-throughput. These newer methods are allowing a more in-depth analysis of the oral microbiome than was previously possible.

1.3.2 *Microscopy and culture methods*

Various forms of microscopy have been used to detect and enumerate oral bacteria, the earliest example of which dates back to the 17th century when Antony Van Leeuwenhoek observed tiny 'animalcules' in his own tooth scrapings with his single-lens optical microscopes (Egerton, 2006). Compound light microscopy techniques were used centuries later to characterise the human subgingival microbiota in healthy sites and in sites with periodontal disease (Socransky et al., 1963, Loe et al., 1965, Listgarten, 1976, Listgarten and Helldén, 1978). These studies identified the different groups of bacteria present in plaque samples based on their morphological characteristics under the microscope. Loe et al. (1965) and Theilade et al. (1966) were able to describe the broad shifts in the predominant morphotypes as plaque matured and gingivitis developed in their longitudinal 'experimental gingivitis' model whilst Listgarten and Helldén (1978) observed differences in the microbiota found at healthy and periodontally diseased sites in the same patients. However, by using light microscopy techniques alone, it was not possible to identify or distinguish the species observed in the samples, only the major morphological groups. The introduction of electron microscopy provided a greater resolution with which to differentiate groups of bacteria morphologically (Saxton, 1973, Nyvad and Fejerskov, 1987) but remained limited in this respect. However, scanning electron microscopy (SEM) enabled a more detailed description of the fine structures formed

by aggregates of bacterial cells within dental plaque such as ‘corn-cobs’ (Saxton, 1973).

The addition of immunological techniques to microscopy, enabled some species to be delineated as well as localised within different sections of plaque samples (Kigure et al., 1995). Fluorescent *in situ* hybridisation (FISH) and confocal microscopy have allowed researchers to visualise the spatial arrangement of specific bacteria within dental plaque (Palmer et al., 2003, Zijnge et al., 2010). Indeed, FISH was used to detect *in vivo* adhesion among *Actinomyces* spp. and *Streptococcus* spp. in early plaque biofilms (Palmer et al., 2003). Furthermore, this technique has allowed previously uncultured taxa to be visualised and enumerated within plaque. Recently, the morphology of uncultivated members of the *Synergistes* phylum was described and estimates of their abundance within subgingival plaque was determined using FISH techniques (Vartoukian et al., 2009).

Cultural methods were used extensively prior to the advent of molecular techniques to study the oral microbiome. Traditionally, bacteria from an oral sample, such as plaque or saliva, were grown aerobically or anaerobically on complex artificial culture media and pure cultures were obtained for identification using conventional phenotypic testing. However, identification of isolates is now increasingly performed using genotypic analysis such as through the sequencing of ‘housekeeping’ genes, particularly the 16S rRNA gene (see following section). Enriched culture media have been used to recover as broad a range of species as possible from a sample and a combination of methods and conditions can give a better representation of species diversity (Vartoukian et al., 2010). When looking to detect specific groups of organisms, selective media can also be employed. For instance, selective media have been useful for isolating specific organisms found to

be associated with dental caries lesions including *Bifidobacterium* spp. and *Lactobacillus* spp. (Marchant et al., 2001, Beighton et al., 2010, Tanner et al., 2011). However, the numbers of useful selective media for isolating members of the oral microbiome are currently limited. Among the most extensive cultural analyses of the oral microbiome were those of Moore and colleagues (Moore et al., 1982, Moore et al., 1987, Moore and Moore, 1994) who cultured plaque samples from health, gingivitis and periodontitis using a variety of different media and incubation conditions. They identified a total of 509 bacterial taxa from the 51,000 isolates obtained from the gingival crevices of 300 individuals (Moore and Moore, 1994). *F. nucleatum* and *A. naeslundii* were the taxa most frequently isolated. From these analyses, they were able to associate specific taxa with health and periodontal disease, and noted the considerable variability in the cultivable bacterial flora between individuals. Of the 35 taxa they associated with gingivitis and/or periodontitis, *F. nucleatum* was reported to be the most likely to initiate periodontal disease. In addition, *F. nucleatum* was found to increase in proportion with increasing disease severity and to be the predominant species present in sites with destructive disease. Other taxa found to be associated with periodontitis included the species *T. denticola* and *P. gingivalis* which were later among the 'red complex' species described by Socransky et al. (1998).

The difficulties associated with using culture to characterise the oral microbiome include the slow growth and fastidious nutritional requirements of many species and, critically, the current inability to cultivate many of the bacteria found in the oral cavity (Socransky and Haffajee, 2005). Early microscopy studies (Loe et al., 1965, Listgarten, 1976) revealed the presence of numerous spirochetes in dental plaque examined from subjects with gingivitis and chronic periodontitis, that were

later only found in very small numbers in cultural studies analysing similar samples (Moore and Moore, 1994). 'Unculturable' or 'as-yet-uncultivated' bacteria, including many of the spirochetes, are defined as those organisms that are yet to grow in artificial media *in vitro* (Vartoukian et al., 2010). It has been previously estimated that approximately half of the human oral microbiome is 'unculturable' (Wade, 2004). In a recent report, 34.4% of the 619 taxa listed in The Human Oral Microbiome Database were uncultivated (Dewhirst et al., 2010). Given the significance of other unculturable bacteria in the pathogenesis of disease, such as *Treponema pallidum* the aetiological agent of syphilis, the unculturable members of the oral microbiome are likely to be involved in disease and should not be overlooked. Attempts to accurately determine the bacterial diversity present in a given sample are hindered by the inherent bias against unculturable bacteria. Consequently, cultural studies alone are insufficient for a comprehensive characterisation of the oral microbiome. Despite these shortcomings, culture techniques remain valuable as they enable researchers to undertake further analysis of a given isolate's biological properties, which can be particularly useful in the case of novel putative pathogens (Tanner et al., 1986). For instance, the antibiotic sensitivity or resistance profiles of isolates can be determined (Kinder et al., 1986) as can their ability to produce virulence factors (Ciborowski et al., 1994). In addition, culture methods enable researchers to detect viable organisms present within a sample as the modern nucleic acid-based molecular methods alone may not distinguish between live and dead cells.

1.3.3 *Molecular methods*

1.3.3.1 Culture-independent sequencing techniques

The introduction of culture-independent molecular techniques has enabled researchers to reveal more of the bacterial diversity present in the oral cavity. Indeed, using these approaches, whole lineages of previously undetected bacteria such as the Divisions TM7 and SR1 have been consistently found to reside in the oral cavity (Dewhirst et al., 2010) Moreover, specific uncultured taxa have been implicated in oral diseases. For example, uncultured phylotypes of *Catonella, Streptococcus, Atopobium*, *Eubacterium*, TM7*, Synergistetes* and *Treponema* have all been associated with chronic periodontitis (Brinig et al., 2003, Kumar et al., 2003, Griffen et al., 2012).

One of the most widely used culture-independent analysis methods has been 16S ribosomal RNA (rRNA) gene cloning and sequencing. The use of the 16S rRNA 'housekeeping' gene for phylogenetic analysis of prokaryotes was pioneered in the late 1970s (Woese and Fox, 1977). This work used the 16S and 18S rRNA gene sequences of prokaryotes and eukaryotes, respectively, to infer the evolutionary relationships of organisms in the form of phylogenetic trees. This led to the division of all life on earth into the three domains *Archaea* (previously *Archaebacteria*) *Bacteria* (previously *Eubacteria*) and *Eucarya*. The *Bacteria* were separated into 11 distinct lineages based on the 16S rRNA gene sequences of the then-cultured bacteria. The 16S rRNA gene has since proven to be ideal for the purposes of classifying and identifying bacteria, because of the combination of highly conserved and hypervariable regions spanning its ~1500 bp length (Clarridge, 2004). The nine hypervariable regions (V1-V9, see Figure 1) of the 16S rRNA gene have a particularly high sequence diversity among different bacterial taxa, thereby enabling

their differentiation. The conserved regions (containing low sequence diversity) allow the design of so-called 'universal', or broad-range, primers that enable PCR amplification and sequencing of a wide range of bacteria. The 16S rRNA gene sequence of a given organism can be used for its identification by comparison to reference 16S rRNA sequences available from online database such as the Human Oral Microbiome Database (HOMD) (Chen et al., 2010), the Ribosomal Database Project (Cole et al., 2009) or the National Center for Biotechnology Information (NCBI) nucleotide database (Altschul et al., 1990). Using a Basic Local Alignment Search Tool (BLAST), for example, the percentage sequence similarity to 16S rRNA gene sequences within the database can be determined and used for taxonomic assignment of the sequence. A percentage similarity threshold is usually applied to determine the species-level identity. However, one limitation of this is that there is no universal consensus percentage similarity threshold for assignment of a sequence to a species, although thresholds of 97, 98.5 or 99% have frequently been applied (Janda and Abbott, 2007).

Despite some limitations, 16S rRNA-based analyses have had a major impact on bacterial taxonomy and resulted in a means of identification of bacteria that is considered more accurate and reproducible than phenotypic testing (Woo et al., 2008). As a result, the 16S rRNA gene is often chosen for use in microbial ecological studies to identify the bacteria present in complex samples. Such studies have most commonly used a cloning and Sanger sequencing method which involves the PCR amplification of 16S rRNA genes directly from a sample using 'universal' primers followed by integration of the amplicons into a plasmid vector by a ligation or topoisomerase reaction and then transformation of competent *Escherichia coli* cells. Clones from the resulting library may then be sequenced using conventional Sanger

sequencing, typically by means of an automated capillary sequencer. The 16S rRNA gene sequences generated can then be compared to a reference database for identification as described above. One major advantage of the 16S rRNA sequencing approach is that both ‘universal’ and specific PCR primers can be designed (de Lillo et al., 2004). This enables characterisation of potentially the entire bacterial community or the identification of specific taxa, or groups, within the community.

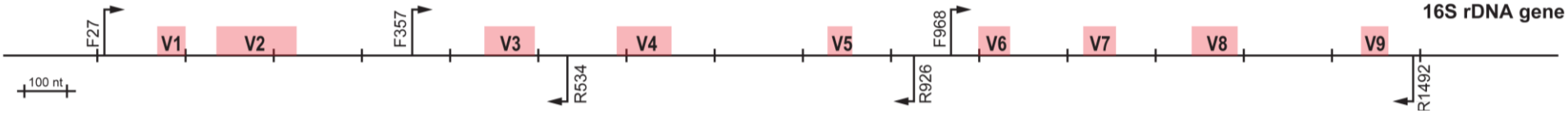

Figure 1: Schematic representation of the 16S rRNA gene. In this representation of the gene the nine hypervariable regions are marked in pink and labelled V1-V9. The positions of commonly used 'universal' primers are also marked. The positions and numbers are based on the *Eschericia coli* 16S rRNA gene. Adapted from "Evaluation of 16S rDNA-based community profiling for human microbiome research" by Ward et al. (2012) under the Creative Commons Attribution License (CCAL).

One of the earliest attempts to use 16S rRNA gene-based phylogenetic analysis for culture-independent investigation of oral bacteria was in the study of Choi et al. (1994), who aimed to investigate the diversity of cultivable and 'unculturable' spirochetes in the gingival crevice of a patient with severe periodontitis. The study was significant in uncovering much greater than anticipated species diversity. A succession of 16S rRNA gene cloning and Sanger sequencing studies followed this, with many focused on determining the specific bacteria associated with diseases of the oral cavity such as dentoalveolar abscesses (Dymock et al., 1996, Wade et al., 1997), dental caries (Munson et al., 2004) and periodontal diseases (Spratt et al., 1999, Dewhirst et al., 2000, Hutter et al., 2003, Kumar et al., 2003, Kumar et al., 2005, de Lillo et al., 2006, Faveri et al., 2008). These studies were able to identify a number of species of unculturable or as-yet-uncultivated bacteria that were associated with disease. Kroes et al. (1999) investigated the bacterial diversity in the subgingival crevice of a single patient with mild gingivitis. This study provided a direct comparison between culture and culture-independent methods and confirmed that the latter method revealed much greater diversity than the former. In addition, 52.5% of 16S rRNA gene sequences from the cloning showed <99% identity to sequences in public databases, indicating that the oral microbiome was poorly defined. A 16S rRNA gene cloning and sequencing study by Sakamoto et el. (2000) compared the bacterial composition of saliva samples from a healthy subject and two periodontitis patients, and found a number of previously uncharacterised and uncultured bacteria. Another similar extensive study by Paster et al. (2001) aimed to determine the bacterial diversity in subgingival plaque of both healthy subjects and patients with periodontitis. The 2,522 clones sequenced were assigned to 347 species-level phylotypes and richness estimates predicted an

additional 68 unseen species. Of the 215 novel phylotypes detected in this study, 33 were cultivable strains not previously characterised and the remaining 182 were represented only by clones; with the vast majority presumed uncultivable. Nine distinct bacterial phyla were detected, with *Firmicutes* most frequently represented and *Spirochaetes* having the most novel phylotypes. In addition, novel phylotypes of the OP11 and TM7 candidate phyla were detected, which had no cultivable representatives. The studies of Kroes et al. (1999), Paster et el. (2001) and Sakamoto et al. (2000), in addition to others, were important as they began to reveal the true extent of the richness and diversity of the oral microbiome.

Whilst considerable contributions to our knowledge of the composition of the oral microbiome have been made through molecular 16S rRNA gene studies using cloning and Sanger sequencing, there are limitations to the approach and hence results from such studies must be interpreted with caution. Biases can be introduced due to the specificity of the 'universal' PCR primers, the number of PCR cycles used, different DNA extraction methods, cloning, and variability in numbers of rRNA operons between species (Suzuki and Giovannoni, 1996, Petrosino et al., 2009, Nossa et al., 2010). Such biases may result in inaccurate representations of different bacterial taxa in a sample. For example, the under-representation of the high G+C content phylum *Actinobacteria* in culture–independent molecular cloning studies when compared to culture has been reported (de Lillo et al., 2006). In addition, oral bifidobacteria which have been associated with dental caries (Beighton et al., 2010) are rarely detected using 16S rRNA cloning and sequencing, which is likely to be due to mismatches for this group with commonly used universal primers (Frank et al., 2008). Divergent results between studies of the oral microbiome in health by Aas et al. (2005) and Bik et al. (2010), in particular the greater numbers of

species of the *Proteobacteria* phylum detected in the latter study, was suggested by the authors (Bik et al., 2010) to be due to the use of different broad-range PCR primers and DNA extraction methods as well as a deeper sequencing effort per individual. As well as biases, other problems associated with Sanger sequencing studies include the high cost and time-consuming nature of the method, and the relatively low depth of sampling which can prevent the detection of species in low abundance.

The last decade saw the introduction of a number of 'next-generation sequencing' (NGS) technologies such as 454 Life Sciences' massively parallel pyrosequencing (Margulies et al., 2005), Illumina/Solexa's sequencing by synthesis and 'bridge amplification' technology (Mitra and Church, 1999) and Applied Biosystems' 'SOLiD' techonology (Sequencing by Oligonucleotide Ligation and Detection) (Mardis, 2008) which have significantly increased the scale and potential of molecular ecological studies of the oral microbiome. Due to the vastly increased sequence output from a single run on an NGS platform, the cost of sequencing per nucleotide base has dropped dramatically compared to several years ago (Voelkerding et al., 2009). The first of the NGS systems to become commercially available was 454-pyrosequencing. This system combines clonal amplification of compartmentalized nucleic acid templates using 'emulsion-PCR' (emPCR) on beads and pico-titer wells for the pyrosequencing of individual templates in parallel (Margulies et al., 2005). 454-pyrosequencing uses a chemistry that is based on the detection of light, following enzymatic reactions that are initiated by the release of inorganic pyrophosphate (PPi) each time a single nucleotide is incorporated into a growing sequence (Ronaghi et al., 1996). A single run of 454-pyrosequencing is capable of generating close to a million sequences, thereby providing much greater

throughput than conventional Sanger sequencing methods using automated capillary electrophoresis (Rothberg and Leamon, 2008). This platform is particularly useful for 16S rRNA gene-based studies of bacterial communities as it provides the longest read lengths of the next-generation sequencing platforms (at the time of writing) enabling sufficient taxonomic resolution to analyse bacterial communities at the species-level. The latest version of the 454 sequencer (GS-FLX+ Titanium series) is reported to produce 1000,000 reads in a single run with an average length of 700 bases (GS-FLX + flier, Roche 454 website). However, with the rapid pace of advancements in existing sequencing technologies such as that of the Illumina MiSeq platform, which now allows paired-end reads of up to 250 bases in length, and the introduction of newer NGS technologies such as Life Technologies' 'Ion-Torrent' and Oxford Nanopore's miniature 'MinION' sequencing system, this may change in due course.

The use of 454-pyrosequencing of 16S rRNA genes for bacterial community analysis greatly improves the depth of coverage (in regards to the number of sequence reads obtainable from a mixed set of PCR amplicons) when compared with Sanger sequencing, enabling detection of rarer, less abundant community members (Petrosino et al., 2009). Furthermore, the absence of a requirement for the cloning step in bacteria, the ability to multiplex numerous samples in a single run through the use of molecular 'barcodes' (Dowd et al., 2008), and the large number of reads obtainable in a single run, gives studies using this approach a significant advantage. 16S rRNA gene pyrosequencing studies of the oral microbiome have, to date, aimed to describe the health-associated oral microbiome (Keijser et al., 2008, Zaura et al., 2009), compare the composition of plaque bacterial communities in health and chronic periodontitis (Griffen et al., 2012, Abusleme et al., 2013), analyse the

bacterial diversity of endodontic infections (Li et al., 2010), describe bacterial communities in the apical root canal (Santos et al., 2011, Siqueira et al., 2011) and assess variations in the composition of the salivary microbiome (Nasidze et al., 2009, Yang et al., 2012). These studies are revealing even greater bacterial diversity in the oral cavity than was previously found by Sanger sequencing-based studies. This was highlighted by Li et al. (2010) who undertook a direct comparison between 454-pyrosequencing and Sanger sequencing on the same seven samples from endodontic infections, resulting in the detection of 13 phyla and 179 genera by the former and eight phyla and 25 genera by the latter. 454-pyrosequencing studies are rapidly adding to our knowledge of the differing compositions of the oral microbiome in health and disease and are increasing the list of bacterial taxa that are associated with oral diseases such as chronic periodontitis (Wade, 2011). One notable recent study compared the bacterial populations in dental plaque from healthy individuals and from individuals with chronic periodontitis and found major differences in their population structure at all phylogenetic levels (Griffen et al., 2012). The investigators identified 53 bacterial species associated with health and 123 with disease and reported a significantly increased bacterial richness and diversity in disease compared to health. The species associated with health and chronic periodontitis as a result of studies using high-throughput sequencing approaches will be discussed in greater detail in a later section.

Limitations of 454-pyrosequencing studies have so far included low phylogenetic resolutions due to short 16S rRNA gene sequence read lengths and over-estimation of phylogenetic diversity resulting from base-calling errors in regions of sequence with homopolymers in 454 sequencing (Quinlan et al., 2008). Base-calling errors occur when the light intensities detected by the instrument, for a

given number of the same nucleotide in sequence, do not accurately represent the actual number of that nucleotide within the homopolymer (Quince et al., 2009). Kunin et al. (2010) investigated the effects of such base-calling errors on 16S rRNA phylotype diversity estimates using a single template belonging to *Escherichia coli* MG1655 and found that sequencing errors inflated the estimates of actual diversity by almost two orders of magnitude. The effects of pyrosequencing errors and short read lengths were evident in the results of an early study of the oral microbiome using the original 454 platform (Keijser et al., 2008). By analysing sequences of <250 bases of a hyper variable region of the 16S rRNA gene, the investigators were unable to reliably classify Operational Taxonomic Units (OTUs) below the genus-level but nevertheless estimated the total species richness in the oral cavity to be between 19,000-26,000 species. Increased read lengths of 454 pyrosequencing and algorithms to correct base-calling errors (Quinlan et al., 2008, Quince et al., 2009) are helping to address these problems. Developers of the 'mothur' software suite (Schloss et al., 2009) for analysis of sequence data demonstrated marked reduction in error rates of their pyrosequencing data sets through the analysis of 90 identical mock community samples (Schloss et al., 2011). Using a combination of methods and by implementing the PyroNoise algorithm into the analysis (Quince et al., 2011) they were able to reduce the total error rate from 0.0060 to 0.0002. The authors reiterated, however, that accurate detection of sequence chimeras (hybrid sequences arising from two or more parent sequences) in sequence data sets remains a challenge and that chimeras are an important source of erroneous reads. Undetected chimeras inflate estimates of species diversity in a given sample as they are incorrectly assigned to unique OTUs or species-level phylotypes. This issue, however, is common to other NGS platforms and is not specific to the pyrosequencing

technology. Furthermore, NGS methods based on 16S rRNA gene sequencing, in common with conventional cloning and Sanger sequencing, are subject to potential biases arising from DNA extraction methods and PCR as described earlier.

With the recent increases in read length (to 250 bases) obtainable on the Illumina MiSeq instrument there has been considerable interest in using this platform for 16S rRNA sequencing studies. One major advantage this platform has over 454-pyrosequencing is the vastly greater number of sequences generated in a single run (24-30 million paired-end reads per run) at a lower cost. By sequencing 16S rRNA gene amplicons of an appropriate size, investigators can design an experiment to ensure that paired-end reads overlap and that sequences can therefore be aligned and joined to produce longer single reads. One study has reported the successful use of the paired-end approach for 16S rRNA sequencing (Bartram et al., 2011), however this was using the shorter 125-base reads available at the time. To date, one study has reported the use of Illumina sequencing for 16S rRNA gene sequencing of the oral microbiome (Lazarevic et al., 2009). However, this study did not use the paired-end approach and the analysis was based on very short reads of <100 bases in length targeting the V5 region of the gene thereby limiting the phylogenetic resolution of the analysis.

In addition to sequencing amplified 16S rRNA genes, investigators have begun to employ metagenomic 'shotgun' approaches on NGS platforms for bacterial community analyses (Xie et al., 2010, Belda-Ferre et al., 2011). By fragmenting extracted DNA, ligating the fragments with sequencing adaptors, sequencing the fragments and finally assembling the sequence reads, investigators can analyse the whole genetic content of bacterial communities. Metagenomic sequencing has the major advantage of providing functional information concerning the bacterial

communities as sequenced genes can be assigned known or putative functions. At the same time, however, 16S rRNA gene reads can be extracted from the data and used for taxonomic assignment of the species present (Belda-Ferre et al., 2011). As this approach does not require PCR amplification, it should also overcome the biases associated with 16S rRNA PCR and sequencing. A drawback of the method, however, is the large yield of genomic DNA required from the starting sample, which may be problematic when small samples are obtained (e.g. in gingival health when plaque levels are generally low). Another approach that has recently been implemented is to use metatranscriptomic analysis to determine the levels of gene expression of oral microbial communities (Frias-Lopez and Duran-Pinedo, 2012). This potentially enables investigators to identify bacterial genes of importance that are up- or down-regulated in disease.

1.3.3.2 Other molecular methods

A number of other nucleic acid-based molecular approaches including PCR, quantitative real-time PCR, and DNA-DNA hybridisation have been used to study the oral microbiome. Studies using these methods have led to some significant findings. For instance, the association of the 'red complex' species (*P. gingivalis*, *T. forsythia* and *T. denticola*) with chronic periodontitis was based on the results of a "checkerboard" DNA-DNA hybridisation technique (Socransky et al., 1994, Socransky et al., 1998, Ximenez-Fyvie et al., 2000). In this method, target bacterial genomic DNA in a sample binds to a set of specific labelled whole genomic DNA probes resulting in detectable signals corresponding to the levels of particular taxa present. One advantage of this technique is that multiple targets can be screened for in parallel, typically around 40. A major limitation of the whole genomic probe approach though, is that probes can only be designed for known cultivable species.

However, 16S rRNA gene sequences can be used to design specific so called 'reverse-capture' checkerboard hybridisation probes (Becker et al., 2002), permitting the detection of uncultivated bacteria. Another limitation applicable to both approaches is that the close genetic relatedness of many oral bacteria can result in hybridisation of non-target species to a given probe (cross-reactions) (Socransky and Haffajee, 2005). This can result in over-estimation of the abundance of a particular species within a community and to false positive results. Both approaches also suffer from the limited numbers of taxa that can be screened for in parallel. With the development of microarray technology, hundreds of labelled probes can be bound to a single glass slide to detect more taxa simultaneously. For example, the Human Microbe Identification Microarray (HOMIM) has been designed to detect 300 of the most prevalent oral bacterial species using reverse capture 16S rRNA probes (Colombo et al., 2009) and has been used in a number of studies of the oral microbiome in both health and disease. Ahn et al. (2011) compared the use of HOMIM to 454-pyrosequencing for the purpose of characterising oral bacterial communities. The detection of bacterial phyla and the most common genera by the two methods were highly correlated. Unsurprisingly though, being a closed-ended approach, HOMIM detected fewer genera overall. However, comparison of the results was not performed at the species-level. An interesting finding based on results obtained in a study using HOMIM complemented with quantitative real-time PCR (qPCR), was that the salivary microbiota appears to be distinct in individuals with pancreatic diseases compared to healthy individuals (Farrell et al., 2011). HOMIM analysis identified 31 taxa that were increased in the saliva of ten patients with pancreatic cancer, relative to ten healthy individuals. Subsequent validation of 16 of these taxa using qPCR in a larger population, found that *Neisseria elongata* and *S*.

mitis were significantly less abundant in patients with pancreatic cancer compared to healthy individuals, whilst *Granulicatella adiacens* was significantly more abundant. Although there was no evidence indicating that these changes in the salivary microbiome were causative, the results did suggest a possibility for applying the detection of such changes to disease diagnosis.

1.4 The oral microbiome in health

1.4.1 *Function of the oral microbiome in health*

The bacterial communities that constitute the normal oral microbiome co-exist with the host in, what is for the large part, a mutually beneficial relationship (Marsh and Percival, 2006). Indeed, disruption of this host-microbiome homeostasis can be problematic and may lead to disease (Darveau, 2010). For example, suppression of the normal bacterial biota as a result of broad-spectrum antiobiotic therapy is a well-recognised risk factor for oral candidiasis (Epstein and Polsky, 1998). This is because the yeasts *Candida albicans* and other *Candida* spp., commonly present in low numbers in the oral cavity, are sometimes able to overgrow and cause infection when the commensal bacterial communities are sufficiently disrupted. In addition, bacteria ordinarily colonising oral surfaces can assist the host by acting as a barrier to colonisation by exogenous pathogens such as *Staphylococcus aureus,* a mechanism termed colonisation resistance (Vollaard and Clasener, 1994). These species may compete with pathogens for available nutrients or attachment sites. Investigators have sought to exploit this by the use of so-called 'effector strains' for replacement therapy in the prevention of dental diseases (Hillman and Socransky, 1987). In the case of dental caries, mutants of the cariogenic bacterium *Streptococcus mutans,* that produce low amounts of acid compared to normal *S. mutans* strains, were proposed as effector strains. Some oral bacteria are capable of producing bacteriocins that may

be inhibitory to potential pathogens or pro-inflammatory organisms (discussed further in a later section). One example of this is bacteriocin production by a strain of the health-associated organism *Streptococcus salivarius* (strain K12). This strain has been investigated for use as a probiotic to prevent and treat halitosis (oral malodour) through its bacteriocins (salivaricins) that have inhibitory activity against a number of obligate anaerobes that produce malodorous volatile sulphur compounds and short chain fatty acids, such as *Solobacterium moorei* and *Eubacterium saburreum,* (Burton et al., 2006). In addition to bacteriocins, commensal bacteria can produce other inhibitory products, such as hydrogen peroxide, that can supress the growth of opportunistic pathogens (Zhu and Kreth, 2012).

Oral commensal bacteria may help to stimulate, develop and regulate the human immune system, as has been well documented in the case of the gut (Cebra, 1999). For instance, one study using a mouse model showed that oral bacteria influenced the host expression of the innate defence mediator Interleukin-(IL)-1β as conventionally reared mice had significantly higher mRNA and protein levels of IL-1β than germ-free mice (Dixon et al., 2004). A study investigating the impact of the probiotic *S*. *salivarius* K12 on the host immune response showed that this strain was able to down-regulate both the secretion of the pro-inflammatory chemokine Interleukin-8 (IL-8) and the activation of the NF-κB pathway (Cosseau et al., 2008). Another study demonstrated that the health-associated species *S*. *mitis*, *S*. *salivarius* and *S*. *sanguinis* lowered IL-8 production in oral keratinocyte cell lines induced by infection with the putative pathogen *Aggregatibacter actinomycetemcomitans* (Sliepen et al., 2009). There is evidence, then, that health-associated bacteria such as *S*. *salivarius* are able to actively promote homeostasis with the host and may help to protect host tissue from damage resulting from the stimulation of the immune

response by pro-inflammatory organisms, or toxins such as lipopolysaccharide (LPS).

Another potentially important function of the oral microbiome is its ability to reduce nitrate (NO_3^-) from the host's dietary intake (Schreiber et al., 2010). After ingestion, approximately 25% of nitrate (NO_3^-) is absorbed through the stomach and enters the entero-salivary circuit where it is subsequently reduced to nitrite (NO_2^-) by bacterial nitrate reductases in the mouth (Webb et al., 2008). This may be beneficial to the host as nitrite is absorbed into human plasma where it is reduced to nitric oxide (NO), which has vasodilatory properties (Lundberg et al., 2008). A recent study demonstrated that the use of a chlorhexidine-containing antiseptic mouthwash over a seven-day period resulted in a decreased oral nitrite production and nitrite levels in plasma, which was correlated to an increase in diastolic and systolic blood pressure compared to an initial seven-day control period (Kapil et al., 2013). If the results of this short duration study are reproduced in studies with confounding variables fully controlled, this might suggest that oral bacteria help to regulate host blood pressure and overly effective oral hygiene may even be inadvertently detrimental to general health.

1.4.2 *Current knowledge of the composition of the oral microbiome in health*

Relatively few studies have characterised the oral microbiome in health, as the emphasis in the past has been on the detection and characterisation of the bacteria associated with dental diseases. However, as noted earlier, investigators are increasingly recognising the importance of obtaining a comprehensive description of the microbiome in health, to better understand the changes that can lead to dental diseases, and to develop new strategies to prevent them (Caglar et al., 2005, Devine and Marsh, 2009, Wade, 2010, Teughels et al., 2011). With recent technological

advances in sequencing methods for the culture-independent characterisation of bacterial communities, a more in-depth analysis of the oral microbiome in health is now possible.

Some early knowledge of the composition of oral bacterial communities in healthy individuals was obtained in 'experimental gingivitis' studies. Loe et al. (1965) and Theilade et al. (1966) examined plaque samples microscopically in order to visualise the predominant bacterial morphotypes present and found that the bacterial communities in healthy individuals, prior to the development of gingivitis, were relatively simple and consisted of predominantly Gram-positive cocci and rods. The later culture-based 'experimental gingivitis' studies of Loesche and Syed (1978) and Moore et al. (1982) showed that health-associated plaque was dominated by *Streptococcus* spp., *Actinomyces* spp. and *Veillonella* spp. In the latter study, 29 species were negatively correlated with the clinical onset of gingivitis, and of these, *S. sanguinis* was detected in the highest numbers. Moore et al. (1994) found that the predominant species isolated from the subgingival crevices of 11 healthy adults were *A. naeslundii*, *S. sanguinis*, *Veillonella parvula*, *F. nucleatum*, and *S. oralis*.

With the introduction of culture-independent methods it became possible to detect and identify the 'unculturable' members of the oral microbiome in health. A summary of the various bacterial taxa that have been associated with health on the basis of a number of different culture-independent 16S rRNA gene sequencing studies, using both conventional cloning / Sanger sequencing and next-generation sequencing methods, can be found in Table 1. Aas et al. (2005) used 16S rRNA gene cloning and Sanger sequencing to describe the bacterial communities present in five healthy subjects on a variety of different intra-oral surfaces including sites on the tongue, buccal fold, hard palate, soft palate, labial gingiva, tonsils, and supra- and

subgingival plaque from tooth surfaces. The investigators analysed a total of 2,589 16S rRNA gene clones, detecting 141 distinct bacterial taxa representing six phyla (*Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria* and TM7). The dominant genera detected in order of prevalence were *Streptococcus, Gemella, Abiotrophia, Granulicatella, Rothia, Neisseria* and *Prevotella*. At the species / phylotype level, the most common organism across all sites and subjects was the *S. mitis / pneumoniae* group. Over 60% of the taxa detected were previously uncultivated phylotypes, highlighting the value of using culture-independent methods in addition to culture, to reveal the bacterial richness and diversity present in the oral cavity. Of the taxa commonly detected, the investigators found some to show a degree of site specificity such as *S. sanguinis* and *Rothia dentocariosa* (detected more frequently in plaque from tooth surfaces) whilst others such as *S. mitis*/*pneumoniae* and *G. adiacens* were found on most or all of the sites. More recently, a study using a similar approach (Bik et al., 2010) sampled 10 healthy subjects at different oral sites (a total of 26 different oral specimens per subject) and detected nine bacterial phyla and 247 species-level phylotypes from the 11,368 16S rRNA gene sequences analysed. Of these species-level phylotypes, approximately 10% were found to be novel. The predominant genera found were in agreement with those found by Aas et al. (2005) but greater numbers of *Proteobacteria,* in particular from the genera *Haemophilus* and *Lautropia,* were detected. The three phyla detected by Bik et al. (2010), but not by Aas et al. (2005), were *Spirochaetes,* OD2 and *Synergistetes*. The authors suggested that the differences between their study and that described by Aas et al. (2005) were likely to be the greater depth of coverage used in their study, as well as different DNA extraction methods and 16S rRNA primers. Significantly, Bik et al. (2010) identified 15 bacterial genera that were found in all 10

individuals, supporting the concept of a 'core' health-associated microbiome. Of the 247 phylotypes seen in total, however, only *Haemophilus parainfluenzae*, *S. oralis*, *S. sanguinis*, *G. adiacens*, *V. parvula*, *Veillonella dispar*, *Rothia aeria*, *A. naeslundii*, *Actinomyces odontolyticus*, *Prevotella melaninogenica*, and *Capnocytophaga gingivalis,* were shared between all individuals. Interestingly, Aas et al. (2005) did not detect the 'red complex' putative periodontal pathogens whilst Bik et al. (2010) found low numbers of all three in three of the ten subjects. This point emphasises the advantage of using techniques that may detect low abundance taxa in bacterial communities, as they may have important roles in the initiation or progression of disease. With the introduction of high-throughput sequencing methods in recent years this has become readily achievable.

High-throughput 16S rRNA gene sequencing has been used in several studies focused on characterising the microbiome associated with oral health (Keijser et al., 2008, Zaura et al., 2009, Lazarevic et al., 2010). Whilst these studies analysed samples with a greatly increased depth of coverage compared with those using conventional Sanger sequencing, phylogenetic resolution was compromised due to the shorter read lengths that were obtained and the difficulties associated with the analysis of the large sequence data sets generated. In the first of these studies, Keijser et al. (2008) analysed 197,600 16S rRNA gene sequences from the pooled saliva samples of 71 healthy individuals and pooled supragingival plaque samples of 98 healthy individuals. Their results showed the presence of a total of 5600 species-level phylotypes in saliva and 10,000 in supragingival plaque (when applying a 3% distance cut-off for species-level OTUs). They estimated that the total species richness was 26,000 species. This constituted a significantly greater diversity and species richness than any previous studies of the oral microbiome had suggested.

However, it is now recognised that this diversity was significantly inflated, likely due to the afore-noted problems relating to the analysis of the short reads containing errors in homopolymer regions (Quince et al., 2009). A later study by the same group aimed to determine if a 'core' health-associated microbiome exists in the oral cavity by analysing the microbiomes of three healthy individuals (Zaura et al., 2009). Of the 6315 unique sequences generated from all samples taken from different intra-oral niches, 1660 were shared between all individuals and these made up 66% of the total number of sequences. At the genus-level or higher, 72% of taxa were shared. These findings, therefore, supported the concept of a 'core' health-associated oral microbiome. At a 3% distance of OTU clustering, 541,630 and 649 species-level phylotypes were detected within the three individual oral cavities respectively, representing significantly lower species richness than was found by Keijser et al. (2008). Zaura et al. (2009) employed more stringent filtering than Keijser et al. (2008) with the requirement that unique sequences appear at least five times in the total data set in order to be included in the final analyses. Problems with this approach though, include the potential for removing rare members of the microbiome and the influence this may have had on estimates of the total shared/'core' microbiome. Phylogenetic analysis was not performed at the species-level due to short average read lengths of 241 bases. However, analysis at the genus-level revealed the most abundant taxa to be *Streptococcus, Corynebacterium, Neisseria, Rothia, Actinomyces, Haemophilus, Prevotella,* and *Fusobacterium*.

A recent cross-sectional pyrosequencing study that compared bacterial communities in the plaque samples of 29 periodontally-healthy individuals to those of 29 patients with chronic periodontitis, was able to identify a total of four phyla, 30 genera and 53 species that were significantly associated with health (Griffen et al.,

2012). An average of approximately 150-175 species (defined at 98% similarity) were found in individual subgingival plaque samples of healthy individuals, a lower richness than previous pyrosequencing studies in health had indicated. The predominant health-associated genera in order of abundance were *Streptococcus*, *Acinetobacter*, *Haemophilus*, *Moraxella*, *Actinomyces*, *Aggregatibacter*, *Granulicatella* and *Rothia*. Due to the longer average read lengths obtained, identifications were also possible at the species-level. *Streptococcus mitis/pneumoniae*, *S. sanguinis*, *Moraxella osloensis*, *Streptococcus intermedius*, *Acinetobacter junii*, *G. adiacens* and *Acinetobacter* sp. RUH1139 were the most abundant species detected in health. Whilst the results of this study confirmed some of the health associations of specific taxa observed by previous studies, the relatively high abundance of the genera *Acinetobacter* and *Moraxella* in health had not been reported previously. It is important to note that when comparing 16S rRNA gene sequencing studies, differences in methodology and sampling may underlie some of the variability in the results. One important consideration is the hyper variable region of the 16S rRNA gene selected for phylogenetic analysis, as this has been shown to impact the inferred membership and structure of the bacterial communities analysed (Nossa et al., 2010). The sensitivity of 16S rRNA gene PCR coupled with deep sequencing means that contamination by bacterial DNA from the environment can also be a problem in high-throughput sequencing studies of the oral microbiome, and potentially an additional factor accounting for variability in the results. For example, PCR reagents themselves may be contaminated with sufficient bacterial DNA to be amplified in the PCR reaction (Grahn et al., 2003).

Table 1: Current knowledge of the composition of the oral microbiota in periodontal health based on studies using 16S rRNA gene sequencing methods. The taxa listed are those that were either found to be predominant in health or that showed a significant association with health when periodontally-healthy individuals were compared to individuals with periodontal disease.

Study	Method / Approach	Species / Phylotypes	Further details
Paster et al. (2001)	16S rRNA gene cloning and Sanger sequencing. Full-length sequences were used for analysis.	*Actinomyces* sp. oral clone EL030, *Corynebacterium matruchotii*, *Streptococcus cristatus*, *Streptococcus intermedius*, *Streptococcus oralis* / *mitis*, *Streptococcus sanguinis*, *Streptococcus* sp. oral isolate H6, *Veillonella dispar* / *parvula*.	Predominant species detected in subgingival plaque of five healthy subjects (detected in ≥ 3 subjects).
Aas et al. (2005)	16S rRNA gene cloning and Sanger sequencing. Full-length sequences were obtained for the analysis.	*Abiotrophia defectiva*, *Actinomyces* spp., *Granulicatella adiacens*, *Granulicatella elegans*, *Rothia dentocariosa*, *Neisseria* spp., *Streptococcus intermedius*, *Gemella haemolysans*, *Streptococcus mitis*, *Streptococcus* clone EK048, *Streptococcus sanguinis*, *Streptococcus gordonii*.	Predominant species detected in supragingival and subgingival plaque of five healthy subjects (detected in ≥ 3 subjects).
Kumar et al. (2005)	16S rRNA gene cloning and Sanger sequencing. Partial sequences were used for the analysis (hypervariable region(s) not specified).	*Abiotrophia adiacens*, *Abiotrophia* sp. oral clone P4PA_155P1, *Campylobacter gracilis*, *Campylobacter showae*, *Capnocytophaga gingivalis*, *Eubacterium saburreum*, *Eubacterium* sp. oral clone OH3A, *Gemella* sp. strain 1754-94, *Rothia dentocariosa*, *Selenomonas* sp. oral clone DSO51, *Streptococcus mutans*, *Streptococcus sanguinis*, *Veillonella* sp. oral clone X042.	Species significantly associated with health based on comparison of subgingival plaque from 15 periodontally-healthy subjects and 15 patients with chronic periodontitis.
Keijser et al. (2008)	16S rRNA gene pyrosequencing of the V6 hypervariable region.	*Actinomyces*, *Campylobacter*, *Capnocytophaga*, *Corynebacterium*, *Fusobacterium*, *Haemophilus*, *Leptotrichia*, *Neisseria*, *Prevotella*, *Rothia*, *Streptococcus*, *Veillonella*.	Predominant genus-level phylotypes in supragingival plaque samples of 98 periodontally-healthy individuals (>2% relative abundance).
Zaura et al. (2009)	16S rRNA gene pyrosequencing of the V5-V6	*Actinobaculum* sp., *Actinomyces* spp., *Capnocytophaga* sp., *Corynebacterium* spp., *Fusobacterium* sp.,	Predominant phylotypes (not identified to species-level in most

Study	**Method / Approach**	**Species / Phylotypes**	**Further details**
	hypervariable regions.	*Granulicatella* sp., *Haemophilus* spp., *Neisseria meningitidis*, *Neisseria* spp., *Porphyromonas catoniae*, *Prevotella* sp., *Rothia* sp., *Streptococcus* spp., *Veillonellaceae* sp..	cases) >1% relative abundance among three periodontally-healthy subjects. Data is for pooled samples of supragingival plaque, saliva, and samples from the cheek, tongue and hard palate.
Bik et al. (2010)	16S rRNA gene cloning and Sanger sequencing. Close to full-length sequences were obtained for analysis.	*Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Capnocytophaga gingivalis*. *Granulicatella adiacens*, *Haemophilus parainfluenzae*, *Prevotella melaninogenica*, *Rothia aeria*, *Streptococcus oralis*, *Streptococcus sanguinis*, *Veillonella parvula*, *Veillonella dispar*	Predominant species detected in pools of supragingival and subgingival plaque of 10 healthy subjects (detected in all 10 subjects).
Huang et al. (2011)	16S rRNA gene pyrosequencing of the V1-V3 region.	*Haemophilus parainfluenzae*, *Lautropia* sp. AP009, *Streptococcus mitis* / *pneumoniae* / *infantis* / *oralis*, *Streptococcus sanguinis*, *Veillonella atypica* / *dispar* / *parvula*.	Species detected in supragingival plaque of three periodontally-healthy individuals (> 1% mean relative abundance) that were significantly associated with health when communities were compared to those of three individuals with gingivitis.
Griffen et al. (2012)	16S rRNA gene pyrosequencing of the V1-V2 and V4 hypervariable regions.	*Acinetobacter junii*, *Acinetobacter* sp. RUH1139, *Actinomyces viscous* / *naeslundii*, *Arthrobacter woluwensis*, *Gemella morbillorum*, *Granulicatella adiacens*, *Moraxella osloensis*, *Streptococcus intermedius*, *Streptococcus mitis* / *pneumoniae*, *Streptococcus sanguinis*.	Species detected in subgingival plaque of 29 periodontally-healthy individuals (>1 % of total sequences) that were significantly associated with health when communities were compared to those of 29 chronic periodontitis patients.
Abusleme et al. (2013)	16S rRNA gene pyrosequencing of the V1-V2	*Actinomyces gerencseriae*, *Actinomyces odontolyticus*, *Actinomyces* sp. HOT 169, *Actinomyces* sp. HOT 170,	Species detected in subgingival plaque of 10 periodontally-healthy

Study	**Method / Approach**	**Species / Phylotypes**	**Further details**
	hypervariable regions.	*Actinomyces* sp. HOT 175, *Actinomyces* sp. HOT 176, *Actinomyces* sp. HOT 177, *Burkholderia cepacia*, *Porphyromonas catoniae*, *Rothia dentocariosa*, *Streptococcus sanguinis*, Unclassified *Xanthomonodaceae* sp., *Veillonellaceae* sp. HOT 155.	individuals that were significantly associated with health when communities were compared to those of 22 chronic periodontitis patients.

Recently, two high-throughput metagenomic studies on samples from healthy subjects have been reported (Xie et al., 2010, Belda-Ferre et al., 2011). Xie et al. (2010) extracted DNA from supragingival and subgingival plaque samples from one healthy volunteer and performed 'shotgun' sequencing on the pooled sample, using both the 454-FLX sequencer and Illumina Genome analyser IIx. After removing contaminating human DNA sequences, the relatively short reads were assembled into longer contiguous sequences (contigs) and mapped to sequenced reference genomes, resulting in approximately 4% of the reads being assigned to sequenced species. The four major species that were matched to the reference genomes were *C. gingivalis*, *Corynebacterium matruchotii*, *Capnocytophaga sputigena* and *Capnocytophaga ochracea*. The authors detected 668 bacterial phylotypes in total, and similar to 16S rRNA sequencing studies the major phyla represented were *Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria* and *Bacteroidetes*. Belda-Ferre et al. (2011) performed a similar, although more extensive, metagenomic study using 454-pyrosequencing on supragingival plaque samples collected from a total of 25 volunteers. The volunteers were divided into groups that had no caries history and were healthy, those that had been previously treated for caries and had a low number of active caries, and finally those that had a high number of active caries and poor oral hygiene. The community composition of the samples was determined both by extracting 16S rRNA gene reads from the data and assigning them to a taxonomy, and by two different methods of assigning the total reads to a taxonomy. The results showed that although the latter approaches confirmed the presence of those taxa detected by the 16S rRNA approach, a broader range of different taxa were identified, including members of the domain *Archaea*. When the authors compared the healthy controls to those with caries, they found that the taxa Bacilli and Gamma-

proteobacteria were enriched in health, whilst *Clostridiales* and *Bacteroidetes* were more abundant in disease. Correspondence analysis based on the 16S rRNA data showed that the genera *Rothia* and *Aggregatibacter* were associated with the absence of caries. In addition, hits to bacterial reference genomes of all the reads (sequence recruitments) showed that an *Aggregatibacter* sp. and *Streptococcus sanguinis* had among the highest levels of recruitment in healthy subjects. Interestingly, following the assignment of reads to functional categories, genes involved in antimicrobial peptide production (bacteriocins), periplasmic stress response, capsular and extracellular polysaccharides, and bacitracin stress response were over-represented in healthy individuals, whilst genes involved in acid fermentation and DNA uptake and competence were over-represented in subjects with active caries.

A major limitation of metagenomic analyses of the oral microbiome currently, is the lack of available reference genomes for the uncultured portion of the microbiome, thus limiting the assignment of contiguous sequences. However, advances in single cell genomics are now leading to the recovery of whole genome sequences of uncultured bacteria. For example, the genome of the organism *Tannerella* sp. BU063 (Human Oral Taxon 286), the closest relative of the putative periodontal pathogen *T. forsythia*, was recently sequenced by isolation of 12 individual cells using flow cytometry (Beall et al., 2014). Interestingly, despite the overall level of genetic similarity between the two species, a number of virulence genes associated with *T. forsythia* (*karilysin, prtH* and *bspA*) were not found in BU063. These differences may help to explain why, despite being very closely related to *T. forsythia*, BU063 is associated with periodontal health (Leys et al., 2002).

1.5 The oral microbiome in periodontal diseases

1.5.1 *Gingivitis*

Gingivitis is defined as inflammation of the gingivae and occurs in response to the accumulation of dental plaque on the teeth and around the gingival margin. The condition is the mildest and earliest form of periodontal disease and, unlike periodontitis, is fully reversible. Clinical signs of gingivitis include increased redness, swelling and bleeding of the gingivae. Gingivitis is extremely common, with the majority of adults being affected during their lifetime. Longitudinal clinical studies have shown that persistent gingivitis is a major risk factor for chronic periodontitis and that inflammation is a pre-requisite for attachment loss (Lang et al., 2009). However, it is important to note that not all individuals or sites with gingivitis develop destructive disease. Studies using an experimental gingivitis model (Loe et al., 1965, Theilade et al., 1966) provided the first convincing evidence that the accumulation of dental plaque elicits gingivitis. They reported that when a group of periodontally-healthy individuals abstained from oral hygiene over a three-week period, all of the individuals developed gingivitis in response to an increase in the amount of plaque. When oral hygiene was resumed and the amount of plaque reduced, gingivitis was reversed. Microscopic examination of the plaque samples showed that the bacterial communities in plaque had become increasingly complex during the period without oral hygiene, and that there had been an overall shift from a microbiota dominated by Gram-positive cocci and rods to one characterised by higher proportions of Gram-negative rods, filaments, fusiforms, spirilla and spirochetes. Hence, these findings suggested that the development of gingivitis was associated with both a change in the composition of plaque and an increase in the total plaque mass. Subsequent experimental gingivitis studies using culture to

characterise the bacterial composition of plaque largely confirmed the earlier findings, and were able to provide information regarding the specific species present. In particular, they reported an increase in the proportions of particular obligate anaerobic species including *Fusobacterium* spp., *Selenomonas* spp., *Prevotella* spp. and *Treponema* spp. as gingivitis developed (Loesche and Syed, 1978, Syed and Loesche, 1978, Moore et al., 1982, Loe et al., 1986, Moore and Moore, 1994, Zee et al., 1996, Tanner et al., 1998). However, these studies also found that Gram-positive rods, particularly *Actinomyces* spp., increased markedly as a proportion of the total cultivable microbiota over time. This led to the suggestion that the proportions of Gram-negative bacteria in gingivitis-associated plaque may have been overestimated by microscopy, possibly due to dead or old cells staining Gram-negative, and that gingivitis was primarily a result of an overgrowth of Gram-positive species (Syed and Loesche, 1978). However, it is well recognised that the relative proportions of particular taxa within oral communities are likely to be misrepresented in culture studies due to the inherent biases.

There have been relatively few studies using molecular techniques to study the microbiota in gingivitis. One study (Tanner et al., 1998) used a DNA probe assay in combination with conventional culture methods to study the microbiota of health, gingivitis, and initial periodontitis. The only species found to be significantly higher in gingivitis-associated plaque than in health-associated plaque, based on the DNA probe data, were *Prevotella nigrescens* and *P. intermedia*. However, the assay only incorporated probes for 13 known oral bacterial species. Using culture, the authors found that subjects with gingivitis had higher levels of Gram-negative bacteria than healthy subjects, and in particular they detected significantly higher proportions of *Bacteroides forsythus* (now *T. forsythia*) in gingivitis. A recent 16S rRNA gene

pyrosequencing study explored bacterial community differences in the saliva and supragingival plaque of healthy individuals and those with gingivitis (Huang et al., 2011). The communities in plaque, but not in saliva, differed significantly in their structure between health and gingivitis and the authors identified 58 gingivitis-associated species-level OTUs. Many of these OTUs were Gram-negative organisms of the genera *Leptotrichia, Selenomonas,* and *Prevotella*. In addition, six gingivitis-associated OTUs were affiliated to the uncultured candidate division TM7. However, the authors were not able to provide species-level assignments in most cases. A further limitation of this study was its cross-sectional design as extensive inter-individual variation has been reported in the oral microbiome.

Whilst the studies discussed above have shown that the composition of plaque in gingivitis differs to that in periodontal health, it remains unclear which species are important in the initiation of gingivitis. In addition to the proliferation of certain species in plaque, it is thought that the general accumulation of bacterial toxins such as lipopolysaccharide (LPS), a component of the Gram-negative cell wall, as well as enzymes and metabolic products contribute to the initiation or exacerbation of the inflammatory response (Lang et al., 2009). Previous work has shown that oral bacteria can induce an inflammatory immune response via the stimulation of Toll-like receptors (TLRs), a part of the innate immune system (Yoshioka et al., 2008). TLRs are capable of recognising various bacterial components (including lipopolysaccharide) and respond by activating signalling pathways that lead to the induction of pro-inflammatory cytokines (Takeda and Akira, 2005). Yoshioka et al. (2008) examined the propensity for supragingival plaque taken from sites with differing plaque, and bleeding on probing (BOP) scores, to activate TLR-2 and TLR-4 in hamster ovary reporter cells. Plaque removed from

sites with higher BOP and plaque scores induced a greater TLR-4, but not -2, response than plaque from sites with lower scores.

Of the species that have been associated with gingivitis, *F. nucleatum* has been proposed as the most likely cause of initial periodontal inflammation, based on extensive cultural analyses showing its uniform presence in the gingival crevice during the initial stages of gingivitis, and its increase in sites with a raised gingival index (Moore and Moore, 1994). An *in vitro* study (Han et al., 2000) has demonstrated the ability of *F. nucleatum* subspecies *polymorphum* and *nucleatum* to attach to, and invade, human gingival epithelial cells, as well as to induce increased production of the pro-inflammatory chemokine Interleukin-8 (IL-8).

1.5.2 *Periodontitis*

Periodontitis is the inflammation and irreversible destruction of the periodontal tissues, or periodontium. If untreated, periodontitis may lead to tooth loss as a result of progressive loss of periodontal attachment. Chronic periodontitis, previously known as 'adult periodontitis', is the most common form of periodontitis, although a distinct form known as aggressive periodontitis (generalized or localized) is notable for its earlier onset and particularly rapid tissue destruction. Periodontitis is defined clinically by the presence of gingival inflammation at a site with loss of connective tissue attachment, accompanied by alveolar bone resorption (Armitage, 1995). The loss of attachment between the tooth and the connective tissue results in the formation of a 'periodontal pocket', which may deepen as the disease progresses. Chronic periodontitis is most commonly detected in adults over the age of 35, but may have started in young adolescents (Clerehugh, 2008). The disease has a complex aetiology with numerous risk factors including poor oral hygiene, smoking, genetic factors, and diseases such as diabetes mellitus (Stabholz et al., 2010). The underlying

cause, however, is the interaction between the dental plaque biofilm forming at the gingival margin and within the gingival crevice, and the host immune response (Darveau, 2010). Both bacteria and the immune response can contribute to tissue destruction through, for example, the production of tissue-damaging proteases (Kuramitsu, 1998, Korostoff et al., 2000).

Although specific bacteria have been strongly associated with chronic periodontitis, it is unknown which organisms are causal and/or contribute to disease progression. This is because species found in greater numbers or more commonly at sites with severe chronic periodontitis may be as a result of the distinct environment found within the periodontal pocket, compared to the healthy gingival sulcus. In particular, the raised pH, lowered redox potential and the abundance of serum glycoproteins from inflammatory exudate, favours the growth of anaerobic, proteolytic organisms such as *Prevotella* and *Porphyromonas* species (Marsh, 2003), which have been previously associated with periodontitis (Socransky et al., 1998). It should be noted that in the case of aggressive periodontitis, however, there is some evidence from two separate populations of a causal role by infection with a single highly leukotoxic clone (JP2) of the organism *A. actinomycetemcomitans* (Haraszthy et al., 2000, Haubek, 2010) in susceptible subjects.

As noted earlier, many studies have attempted to elucidate the microbial aetiology of periodontitis using a variety of different methods. With the advances in culture-independent molecular techniques in recent years, this has led to a long and expanding list of organisms that are periodontitis-associated (Wade, 2011). Among the most consistently associated organisms are members of the so called 'red complex' species (*P. gingivalis*, *T. forsythia* and *T. denticola*) which were strongly associated with increased clinical measures of bleeding on probing and pocket depth,

and with the presence of each other, in a DNA-DNA probe hybridisation-based study targeting 40 previously cultured organisms (Socransky et al., 1998). Other organisms associated with increased pocket depth, but to a lesser extent, in the same study fell into the 'orange complex' and included *F. nucleatum* (subspecies *nucleatum, polymorphum,* and *vincentii*), *Fusobacterium periodonticum, P. intermedia, P. nigrescens, P. micra* (*Peptostreptococcus micros*), *Campylobacter gracilis, Campylobacter rectus, Campylobacter showae, E. nodatum*, and *S. constellatus*. A later study examining the microbial diversity of subgingival plaque in health, chronic periodontitis, refractory periodontitis (periodontitis which has not responded to therapy), human immunodeficiency virus-associated periodontitis and acute necrotizing ulcerative gingivitis used an open-ended 16S rRNA gene cloning and sequencing approach and uncovered a greater bacterial richness than had been previously detected, with 40% of the sequences identified as belonging to novel phylotypes (Paster et al., 2001). In addition, members of the 'red complex' were found in relatively small proportions of the total microbiota compared to culture studies. It was therefore recognised, that there was the potential for novel or rare taxa, particularly those found only at disease sites in that study, to have an important role in periodontitis. Kumar et al. (2003) designed specific 16S rRNA PCR primers to detect and determine the prevalence ratios of a total of 39 novel uncultured phylotypes and named species across 66 healthy subjects and 66 periodontitis patients. From the resulting data the phylotypes *Deferribacteres* (now *Synergistetes*) clone D084 and clone BH017, *Bacteroidetes* clone AU126, *Megasphaera* clone BB166 (now *Anaeroglobus geminatus*), OP11 (now SR1) clone X112 and TM7 clone I025 were all associated with periodontitis. The authors also confirmed the association of the 'red complex' with periodontitis, and revealed new periodontitis-

associations for the named species *Eubacterium saphenum*, *Porphyromonas endodontalis*, *Prevotella denticola*, *Cryptobacterium curtum* and *Treponema medium*. As the study used a PCR method that only detected the presence of specific taxa and did not provide information regarding their numbers or proportions in plaque, the same group followed this study up by using a 16S rRNA gene cloning and sequencing approach (Kumar et al., 2005). Plaque from 15 healthy subjects and 15 subjects with chronic periodontitis were compared. Among the 25 phylotypes associated with periodontitis, *Peptostreptococcus* (*Parvimonas*) clone BS044, *Filifactor alocis*, *Peptostreptococcus* clone CK035 (*stomatis*), *Megasphaera* clone BB166 (*Anaeroglobus geminatus*), and *Desulfobulbus* clone CH031 were the five most strongly associated. Of the 'red complex' species, only *T. forsythia* was associated with periodontitis. Interestingly many of the bacteria associated with periodontitis were Gram-positive species, in contrast to earlier culture and DNA probe studies that had shown a shift towards a Gram-negative dominated community in disease. However, it should be noted that only 100 clones were analysed per sample and many low abundance species may not have been detected. A later study using 454-pyrosequencing of 16S rRNA genes compared the bacterial profiles of plaque in healthy subjects with those in shallow and deep sites of subjects with periodontitis (Griffen et al., 2012). Several thousand sequences were analysed per sample, allowing a more comprehensive analysis of the bacterial communities present and a greater power with which to detect differences between health and disease. Of the 123 species that were significantly more abundant in periodontitis *F. alocis*, *P. gingivalis*, *T. denticola*, *T. medium* and *Lachnospiraceae* clone JM048 (*Lachnospiraceae* [G-8] sp. HOT500) showed the strongest disease association. Other important observations of this study were that the bacterial communities were

significantly more diverse in periodontitis than in health and that there was a significant shift towards higher levels of Gram-negative organisms in disease. Another recent high-throughput sequencing study compared the bacterial community composition of subgingival plaque from two individuals with periodontitis with that of three healthy individuals (Liu et al., 2012). 16S rRNA gene sequencing was used to identify the bacteria present whilst a metagenomic approach was used to examine functional differences. The authors observed a taxonomic shift from a Gram-positive to Gram-negative dominated community in periodontitis and found the genera *Selenomonas*, *Prevotella*, *Treponema*, *Tannerella*, *Haemophilus* and *Catonella* to be enriched in periodontitis compared to health. However, no disease-associations at species-level were determined. The authors were, however, able to delineate a number of metabolic pathways that were enriched in disease including pathways for fatty acid metabolism, acetyl coenzyme A degradation, aromatic amino acid degradation, ferrodoxin oxidation, and energy-coupling factor class transporters, which were reported to be suggestive of a parasitic microbial lifestyle. Furthermore, a number of virulence factors were enriched in disease including type IV secretion systems, conjugative transposons and the Lipid-A of lipopolysaccharide biosynthesis. One recent study used high-throughput sequencing to examine longitudinal bacterial community shifts in the supragingival plaque and saliva of 19 periodontitis patients before and after periodontal therapy (Yamanaka et al., 2012). Interestingly, the richness and diversity of the bacterial communities in plaque, but not in saliva, were significantly reduced post-therapy compared to pre-therapy. In addition they found that the genus *Corynebacterium* increased significantly in relative abundance post-therapy, whilst the genera *Fusobacterium* and *Kingella* decreased significantly. At the species OTU level, a number of OTUs were identified

as having dissipated post-therapy especially among the genera *Tannerella*, *Porphyromonas*, *Leptotrichia* and *Capnocytophaga*.

Efforts have been made to study the pathogenicity and virulence factors of a number of the hitherto identified putative periodontal pathogens, particularly among the 'red complex', in an attempt to establish their role in eliciting or exacerbating inflammation and/or tissue destruction. Perhaps the most frequently studied is *P. gingivalis*, possibly due to the relative ease with which it can be grown in the laboratory compared to other periodontitis-associated organisms. Studies have shown that this organism possesses an array of virulence factors including cysteine-proteinases (gingipains), fimbriae, lipopolysaccharide (LPS), and outer membrane vesicles (OMVs) (Lamont and Jenkinson, 1998), that enable it to adhere to, and invade, oral epithelia, and subvert the host immune response (Hajishengallis, 2011). Work using a murine animal model found that *P. gingivalis* infected germ-free mice did not develop bone loss, where as normal specific-pathogen free mice colonized with *P. gingivalis* did (Hajishengallis et al., 2011). Furthermore, the authors found that introduction of *P. gingivalis* into the commensal microbiota resulted in an increase in periodontal bone loss. This was accompanied by a shift in the composition of the commensal microbiota and a general increase in the microbial load. These results led to the proposal that *P. gingivalis* is a 'keystone pathogen' which, even at low abundance, can modulate the host complement system, causing alterations to the amount and composition of the commensal oral microbiota and a resulting breakdown in host-microbial homeostasis (Darveau et al., 2012).

The pathogenicity and virulence factors of *T. forsythia* and *T. denticola* have been less well studied partly due to their fastidious growth requirements. Nevertheless, a number of studies in animal models have revealed their pathogenic

potential (Sharma et al., 2005a, Lee et al., 2009). Oral inoculation of mice with *T. denticola* resulted in significantly greater bone resorption compared to mock-infected mice (Lee et al., 2009). Sharma et al. (2005a) demonstrated that infection of specific pathogen free mice with wild-type *T. forsythia* induced alveolar bone loss, while a *T. forsythia* mutant unable to express a cell surface-associated and secreted leucine-rich-repeat protein (BspA) did not. These results indicated that BspA is likely to be an important virulence factor of *T. forsythia*. Furthermore, it has been shown that BspA induces the production of pro-inflammatory cytokines and chemokines that may contribute to bone resorption (Hajishengallis et al., 2002). The C-terminal region of the BspA protein contains a conserved domain that is also found in *P. gingivalis*-associated proteins, including its gingipains. This C-terminal domain is recognised by components of the cell's glycosylation and outer membrane secretory systems (Nguyen et al., 2007). Other putative virulence factors identified in *T. forsythia* include the glycosidases SiaHI and NanH (Sharma, 2010). Glycosidases are able to hydrolyze glycosidic bonds in glycoproteins resulting in the release of mono- or oligo-saccharides which can provide a nutrient source for bacteria. For example, the SiaHI and NanH sialidases of *T. forsythia* can remove the terminal sialic acid residues from glycoproteins present in saliva and gingival crevicular fluid (Thompson et al., 2009). It is thought that, in addition to providing a carbon source, sialidases can expose cryptic-receptors, or 'cryptitopes' to enable adherence to oral epithelial cells (Honma et al., 2011). Recent work has shown that the surface layer (S-layer) of *T. forsythia*, which consists of glycoproteins that mediate adhesion to gingival epithelial cells (Sakakibara et al., 2007), may also modulate the host immune response. Sekot et al. (2011) found that wild-type *T. forsythia* induced a significantly greater production of pro-inflammatory mediators than an S-layer

deficient mutant in gingival fibroblasts and macrophages. This suggested that the S-layer may provide *T. forsythia* with a means of evading the host's innate immune response.

Interestingly, members of the 'red complex' have demonstrated synergy in terms of their virulence (Orth et al., 2011). In one notable study, rats infected with all three members exhibited significantly greater alveolar bone resorption than mice infected with only one (Kesavalu et al., 2007). These studies indicate that the interaction and co-operation between members of the oral microbiome likely play a key role in the development or progression of periodontitis.

1.6 New approaches to maintain periodontal health

1.6.1 *Conventional approaches for the prevention and control of periodontal diseases*

Current approaches to prevent and control periodontal diseases aim to remove, or reduce, bacteria residing in dental plaque biofilms, usually using a combination of professional tooth cleaning and oral hygiene methods. These oral hygiene methods include tooth brushing, interdental cleaning and the use of mouthrinses containing anti-microbial compounds such as chlorhexidine or essential oils. For the treatment of severe periodontitis, administration of systemic antibiotics and/or surgical therapy is sometimes appropriate (Wang and Greenwell, 2001). A major problem associated with the plaque control approach to preventing periodontal disease, is that many individuals do not regularly practise oral hygiene to a standard sufficient to restrict plaque to levels that are compatible with periodontal health. This is in part owing to the relatively high level of motivation and skill required to achieve adequate plaque control (Drisko, 2001). Consequently, gingivitis is highly prevalent among adults.

Another limitation of current oral hygiene approaches is that they are indiscriminate in their elimination of plaque bacteria, which, given the benefits conferred by many of the commensal species present, as discussed earlier, is potentially detrimental to the host. Owing to these limitations alternative strategies to prevent and/or treat periodontal diseases are desirable.

1.6.2 *Oral probiotics and prebiotics*

The recognition that periodontal diseases result from ecological perturbations to the normal oral microbiome in health (Marsh, 2006), has led to an increased interest in developing therapeutic approaches that aim to maintain, or restore, the microbiome in/to a health-associated state (Filoche et al., 2010, Wade, 2010). These approaches may achieve this by directly influencing the composition of the microbiome so as to encourage the proliferation of health-associated bacteria and/or prevent the growth of putatively pathogenic bacteria. This may be possible through the intake of oral probiotics or prebiotics (Caglar et al., 2005, Teughels et al., 2011). Probiotics are defined as live microorganisms that exert a beneficial effect on host health when they are administered in adequate amounts (Sanders, 2008). The idea that live microorganisms may be used to benefit host health was brought to attention early in the 20th century as a result of Elie Metchnikoff's hypothesis that the health and longevity of Bulgarian peasant populations was due to their daily consumption of fermented dairy products. One of the organisms thought to be responsible for exerting this health benefit was the lactic acid-producing bacterium *Lactobacillus bulgaricus,* identified earlier by Stamen Grigorov, and used as a started culture for the production of yoghurt (Anukam and Reid, 2007). To the present day, species of lactobacilli are among the most commonly used probiotic organisms, along with other lactic acid-producers of the genera *Bifidobacterium* and *Streptococcus*. Some

of the species used include *B. bifidum, B. breve, B. lactis, B. longum, L. acidophilus, L. bulgaricus, L. casei, L. reuteri, L. rhamnosus*, and *S. thermophilus*. However, some non lactic acid-producing bacteria, including a strain of *Escherichia coli* (Nissle 1917) (Henker et al., 2007) as well as the yeast *Saccharomyces boulardii* (Dinleyici et al., 2012), have been used with beneficial effect. Probiotic organisms are frequently provided via inoculation into dairy products such as milk-based drinks, yoghurts and cheeses.

The currently available probiotics have largely been assessed for their action on the gastro-intestinal tract, particularly in the prevention and treatment of inflammatory bowel disease, rotavirus-associated diarrhoea, antibiotic-associated diarrhoea and bacterial gastroenteritis (Reid et al., 2003). There are a number of different means by which probiotic organisms are thought to exert their beneficial effects for prevention and treatment of intestinal tract diseases. These organisms may, for example, directly inhibit gastrointestinal pathogens through the production of inhibitory substances such as lactic acid, deconjugated bile acids and bacteriocins, or prevent their adhesion to mucosal surfaces (Oelschlaeger, 2010). In the case of inflammatory bowel disease, probiotics may play a beneficial role by down-regulating the production of pro-inflammatory cytokines (Damaskos and Kolios, 2008). Prebiotics are "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host well-being and health" (Roberfroid, 2007), and have thus far been mostly non-digestible oligosaccharides such as fructo-oligosaccharides (FOS) (Caglar et al., 2005). The specific components of the gastrointestinal tract stimulated are predominantly *Lactobacillus* spp. and *Bifidobacterium* spp., which are then able

to exert their beneficial effects such as enhancing colonisation resistance to gastrointestinal pathogens (Gibson et al., 2005).

The use of probiotics and/or prebiotics for the promotion of oral health has been less well studied than for the gastrointestinal tract. One possible reason for this is that the aetiologies of common dental diseases, particularly periodontal diseases, are relatively poorly defined. In addition, the ability to obtain a sufficiently comprehensive knowledge of the oral microbiome in health, in order to inform pro- or pre-biotic strategies, has only been made possible recently following technological advances in DNA sequencing. Most of the early efforts to use probiotics or probiotic-like approaches in the oral cavity focussed on the potential of so-called 'effector strains' for replacement therapy as a means of preventing dental caries. One group developed genetically modified strains of *S*. *mutans* that were less cariogenic (lower acid production) than wild type *S*. *mutans* strains for this purpose (Hillman and Socransky, 1987). The ability of these strains to produce bacteriocins that were inhibitory to other mutans-streptococci (Hillman et al., 1984), enabled them to colonise plaque effectively by out-competing rivals for the same ecological niche (Hillman et al., 1985b). Recent work by Hillman et al. (2009) evaluated the ability of a lactate dehydrogenase-deficient mutant of *Streptococcus rattus* (JH145), a close relative of *S*. *mutans*, to compete with, and reduce, the numbers of *S*. *mutans* in a rat model. They showed that animals treated once daily with JH145 showed a significant dose-dependent decrease in the total proportions (of total cultivable flora) of an *S*. *mutans* strain (NG8), with which the animals had been previously infected. However, the authors did not report any effect on the development of caries. It is important to note that reducing the levels of *S*. *mutans* in the oral cavity alone may not be sufficient to prevent caries as it is now well recognised that caries has a complex

multi-factorial and polymicrobial aetiology with different *Actinomyces* spp., *Bifidobacterium* spp., *Lactobacillus* spp., and *Streptococcus* spp., as well as yeasts, all being involved (Nyvad et al., 2013).

Oral bacteria that produce bacteriocins, bacteriocin-like inhibitory substances (BLIS) or other inhibitory agents that are active against putative oral pathogens have been recognised for their potential use as oral probiotics (Wescombe et al., 2009). Bacteriocins are ribosomally-synthesised antimicrobial peptides produced by a bacterial strain that are inhibitory towards other strains or species of, often closely-related, bacteria (Cotter et al., 2005). Strain K12 of the indigenous oral health-associated bacterium *Streptococcus salivarius,* has been shown to produce the bacteriocins salivaricin types A2 and B (Hyink et al., 2007), which are inhibitory to a range of other Gram-positive organisms. This strain has shown particularly potent inhibition of the pathogen *Streptococcus pyogenes,* infection with which can result in pharyngitis, tonsillitis and acute otitis media (AOM) among others, leading to the proposal for its use as a probiotic (Tagg, 2004) A recent study demonstrated that a group of adults with recurrent streptococcal pharyngitis given the "BLIS K12" probiotic over a 90-day period had an 80% reduction in their subsequent episodes of streptococcal pharyngeal infection, over a 6-month period (Di Pierro et al., 2013). *S. salivarius* K12 has also been proposed as a therapeutic agent for oral malodour, or halitosis, due to its ability to inhibit species producing malodorous compounds, including black-pigmented Gram-negative anaerobes identified as *Prevotella* spp. (Burton et al., 2006). Indeed, daily dosing of halitosis subjects with *S. salivarius* K12 resulted in a reduction in oral malodour parameters in most individuals, seven and 14 days after commencement. Another strain of *S. salivarius*, M18 (formerly strain Mia), has shown potential for application as an anti-caries probiotic as it carries a

mega-plasmid encoding the bacteriocins – salivaricins A2, 9 and MPS, and displays potent inhibitory activity against mutans-streptococci (Heng et al., 2011). In addition, BLIS activity of a number of other bacteria against putative oral pathogens has been reported. For instance a bacteriocin from *Lactobacillus paracasei* HL32 has been described that is inhibitory to *P. gingivalis*, although its ability to antagonise other oral bacteria was not tested (Pangsomboon et al., 2006). Another study reported bacteriocin production by a strain of the Gram-negative bacterium *Capnocytophaga ochracea*, which was strongly inhibitory towards streptococci including *S. mutans* (Nakamura et al., 1992). In addition, a bacteriocin named nigrescin has been described in a strain of *Prevotella nigrescens* (Kaewsrichan et al., 2004). This bacteriocin was found to inhibit the growth of a range of species associated with chronic periodontitis including *P. gingivalis, T. forsythia* and *P. intermedia*, as well as species of *Actinomyces*.

Various oral bacterial species are also capable of producing other compounds or substances that are antagonistic towards different bacterial taxa, including hydrogen peroxide and lactic acid (Teughels et al., 2011). Oral streptococci, in particular *S. mitis*, *S. sanguinis*, *S. oralis*, *S. gordonii* and *S. sobrinus*, have been shown to produce hydrogen peroxide *in vitro* (Garcia-Mendoza et al., 1993). One study examined the effect of hydrogen peroxide production by *S. sanguinis* on the colonization of rats with the putative periodontal pathogen *A. actinomycetemcomitans*. Gnotobiotic rats infected with a hydrogen peroxide-producing strain of *S. sanguinis* resulted in a 45-fold greater reduction of *A. actinomycetemcomitans* than those infected by a mutant of the same strain unable to produce hydrogen peroxide (Hillman and Shivers, 1988). Certain putative periodontal pathogens, including *P. gingivalis*, are known to be sensitive to changes

in pH, and *P. gingivalis* in particular was unable to grow at a pH of less than 6.5 in an *in vitro* study. Therefore, acid production by lactic-acid bacteria, such as lactobacilli, and the resultant pH changes in the local environment can inhibit the growth of periodontitis-associated taxa. A study of *Lactobacillus* spp. in healthy individuals and chronic periodontitis patients found that obligately homofermentative species, which produce more acid than heterofermentative species, were significantly more prevalent in health and were able to inhibit periodontitis-associated taxa, including *P. gingivalis, P. intermedia,* and *A. actinomycetemcomitans, in vitro* (Koll-Klais et al., 2005). In addition, they found that the *in vitro* antagonistic activity was reduced when the inhibition assays were repeated on media without glucose and *P. intermedia,* which can grow at a pH of 5, was less sensitive to inhibition than *P. gingivalis*. These observations suggested that much of the inhibition detected was due to the production of lactic acid. A problem associated with the potential use of acidogenic bacteria such as lactobacilli and bifidobacteria as oral probiotics, is that these organisms are themselves considered to be caries-associated (Nyvad et al., 2013).

Another means by which oral probiotic bacteria may exert a beneficial effect on the host is through the modulation of the immune response of oral epithelial cells, so as to 'dampen' inflammation. As highlighted earlier, one study demonstrated that *S. salivarius* K12 was able to down-regulate secretion of the inflammatory chemokine Interleukin-8 (IL-8) and activation of the nuclear factor-kappa B (NF-κB) pathway. Zhang et al. (2008) found that *Streptococcus cristatus* was able to attenuate the production of IL-8 induced by *F. nucleatum* subspecies *nucleatum, polymorphum* and *vincentii* infection of oral epithelial cells *in vitro*. *S. sanguinis, S. parasanguinis, S. mitis, S. gordonii, S. mutans,* and *S. oralis* were also found to have similar

properties. Further work showed that the precise underlying mechanism was the inhibition of the nuclear translocation of NF-κB that had been stimulated by the *F. nucleatum* strains investigated. These studies suggested, then, that the oral viridans streptococci can reduce the pro-inflammatory immune response induced by certain putative periodontal pathogens in the oral cavity, and as such may be useful in probiotic applications for periodontal therapy. However, there is currently very limited evidence of their beneficial effect *in vivo*.

1.6.2.1 Clinical trials of oral probiotics

There have been relatively few *in vivo* clinical studies aimed at determining the efficacy of oral probiotics for the prevention and treatment of dental diseases. Whilst some of the results from initial pilot studies performed on relatively small cohorts are promising, concerns have been expressed over the lack of appropriate randomization, blinding and control groups in many cases (Devine and Marsh, 2009, Teughels et al., 2011). It is also important to remember that a randomised placebo-controlled clinical trial seeks to establish whether there are significant differences between test and placebo-control groups that might be attributable to the test procedure. Although there may be differences over time within the test or placebo groups, these within group changes cannot be interpreted as evidence that the test agent has been effective.

With regards to the application of oral probiotics for dental caries, several studies have attempted to determine the effect of various probiotic strains on the total counts of mutans-streptococci present in saliva and occasionally in plaque. For example, in one study the daily administration of *Lactobacillus reuteri,* either as a tablet or by drinking water through a straw containing the organism, resulted in a statistically significant reduction in mutans-streptococci, but not lactobacilli, over a

three-week period, in contrast to placebo controls which did not (Caglar et al., 2006). However, no comparison of the straw and tablet groups with the placebo control group was reported after baseline. Similarly, a study comparing the longer term effects on caries and the risk of caries in a cohort of 594 children (1-6 years of age) given milk containing a strain of *Lactobacillus rhamnosus,* with those given normal milk, over a seven month period, found that those children given the probiotic-supplemented milk had reduced mutans-streptococci counts (Nase et al., 2001). Although the incidence of caries was slightly lower in the probiotic group compared with controls, there was no significant difference between groups. However, the difference almost reached statistical significance in children aged 3-4 years, although there was no difference in caries incidence with probiotics in the older or younger children.

Clinical studies assessing the benefits of probiotics to prevent or treat gingivitis and chronic periodontitis have mostly been performed using *Lactobacillus* spp. as the probiotic organism(s). As mentioned earlier, the use of acidogenic lactobacilli is potentially problematic owing to their role in dental caries. In addition, probiotics selected for application in dental disease are frequently species used for gastrointestinal applications. Probiotic species that are not members of the normal oral microbiota may be less likely to colonise and persist in dental plaque biofilms than indigenous species (Devine and Marsh, 2009). Krasse et al. (2006) provided oral hygiene advice for patients with moderate and severe gingivitis and then either administered two different *L. reuteri* strains or a placebo via chewing gum twice daily for two weeks. At the end of the trial period, the gingival index had fallen in all groups. One of the probiotic groups, however, experienced a significantly greater reduction in gingival index than the placebo group. The plaque index fell

significantly in both probiotic groups but not in the placebo group and it is difficult to explain why there had been improvements in the gingival health of the placebo group in the absence of improved plaque control or the probiotic. A later study using *L. reuteri* probiotic chewing gum in a group of individuals with moderate gingival inflammation attempted to determine a dose-dependent effect upon bleeding on probing (BOP), and the levels of five inflammatory mediators in gingival crevicular fluid (GCF) (Twetman et al., 2009). The authors recorded significantly decreased BOP and GCF after two weeks in groups given the probiotic but not in a placebo group. However this effect was no longer demonstrated after 4 weeks. In the group receiving the highest probiotic dose (2×10^8 CFUs per day), significant reductions in the concentrations of two of the inflammatory mediators: IL-8 and tumour necrosis factor-alpha (TNF-α), were detected although differences in the volume of GCF were not taken into account.

Other groups have focussed their attention on the clinical efficacy of strains of *Lactobacillus salivarius*. The strain *L. salivarius* WB21, originally prepared for use as a probiotic to promote gastrointestinal health, was evaluated for its application as a potential oral probiotic in a clinical trial conducted by one group of investigators (Shimauchi et al., 2008, Mayanagi et al., 2009). The authors administered this strain, and a placebo, in tablet form to two randomly assigned test and placebo groups of smokers and non-smokers, defined as individuals without severe periodontitis, three times a day for eight weeks and assessed the impact on clinical periodontal parameters. Following randomisation at baseline, patients within the placebo group had lower pocket depths and bleeding scores than the experimental group and the differences almost reached statistical significance (P=0.06 and P=0.07 respectively). At the end of the trial period both the placebo and test group showed a reduction in

bleeding on probing, gingival index, probing pocket depth and plaque index but there were no significant differences between test and placebo groups. In the very small number of current smokers there were greater reductions in plaque index and probing pocket depth in the probiotic group compared to the placebo group. Mayanagi et al. (2009) examined the impact that the probiotic had on the numbers of five selected periodontal pathogens within supragingival and subgingival plaque samples, collected in the same study. The authors reported a statistically significant reduction in the numerical sum of these organisms after four, but not eight, weeks in the group given the probiotic, compared to a placebo control group. Taken individually, only one of the five organisms (*T. forsythia*) was significantly reduced after four and eight weeks of taking the probiotic. A recent study examined the impact of probiotic lozenges of *L. reuteri* on inflammation and the plaque biofilm of 18 healthy subjects during experimental gingivitis (Hallstrom et al., 2013). In contrast, to the promising beneficial effects exerted upon gingival inflammation and microbial composition described by earlier studies using *L. reuteri* (Krasse et al., 2006, Twetman et al., 2009), no differences were observed between the test group and the placebo group in terms of the concentrations of certain inflammatory mediators and the amount and microbial composition of plaque. The investigations using lactobacilli, described above, have only considered the effects of probiotics on superficial gingival inflammation and not periodontitis. Once periodontal pockets have formed it has proved very difficult to introduce medicaments of any kind beneath the gingival margin without professional applications.

Very few studies have examined the *in vivo* efficacy of probiotic organisms other than *Lactobacillus* spp. on gingivitis or periodontitis. However, two studies (Teughels et al., 2007, Nackaerts et al., 2008) used a Beagle dog model to determine

the effect of strains of *S*. *salivarius*, *S*. *mitis*, and *S*. *sanguinis* on clinical parameters of inflammation and on the composition of the subgingival microbiota. Teughels et al. (2007) artificially created periodontal attachment loss in the dogs and administered the probiotic mixtures directly into the pockets. The study used a "split mouth" design and this could have introduced the possibility of probiotic bacteria applied to test areas over-spilling to control regions. Plaque samples were taken from experimental sites and analysed by culture up to 12 weeks following baseline, without any provision of oral hygiene. The greatest reduction in counts of anaerobic and black-pigmented bacteria occurred in sites given repeated applications of the probiotic mixture after baseline. In addition, these sites showed the most pronounced reduction in bleeding on probing. However the only significant difference between bleeding in test and control regions occurred with multiple applications of probiotic compared with the root debridement alone and the probiotic application had no effect on pocket depths. In the same study Nackaerts et al. (2008) reported the effect of repeated application of probiotic mixture on the bone density and alveolar bone level over 12 weeks. However, only a very limited number of radiographs were of sufficient quality for analysis because the dogs moved during the radiographic examination. The authors reported a significant increase in bone density in the probiotic group, whilst the group not given the probiotic mixture did not show a significant increase. Similarly there was a significant increase in alveolar bone gain in the probiotic group but not in the control group. However, no statistical analysis comparing test and control sites was reported and therefore it is not possible to conclude whether the probiotic was likely to have accounted for the differences. The claimed and possibly beneficial effects of the *Streptococcus* spp. mixtures reported in these Beagle dog studies are yet to be shown in human trials. One uncontrolled

human report claims to test the efficacy of a mouthwash containing three strains of *Streptococcus*: *S*. *oralis* strain KJ3sm, *S*. *uberis* strain KJ2sm, and *S*. *rattus* JH145 (Zahradnik et al., 2009). The authors found that twice daily rinsing with the mouthwash over a four-week period lowered counts of the caries-associated organism *S*. *mutans* in saliva and the periodontitis-associated organisms *P*. *gingivalis* and *C*. *rectus* in subgingival plaque. However, this could have been a purely chance finding because results were not statistically significant, a small number of subjects were recruited and there was no control/placebo group.

Taken together, the clinical studies conducted to date have provided little evidence for the efficacy of current probiotic strains (predominantly lactobacilli) in the prevention and treatment of periodontal disease. This may in part be because many of the studies were not adequately controlled, were performed on a relatively low number of subjects, and statistical comparisons between test groups and placebo control groups were rarely reported. Many of the significant effects reported, both microbiological and clinical, were changes within a group rather than differences between the test and placebo groups. In addition, the strains chosen may not have been the most appropriate for use as probiotics for periodontal disease. Many of the strains tested were originally intended for use in the distal gut and may not necessarily confer a beneficial effect in the oral cavity. It would therefore be useful to demonstrate, prior to performing clinical studies, a potential beneficial mechanism such as antimicrobial activity against putative oral pathogens and/or anti-inflammatory properties.

1.7 Oral bacterial commensals of the genus *Neisseria*

1.7.1 *Neisseria and the oral microbiome*

The aerobic, or facultatively anaerobic, Gram negative, coccobacilli of the genus *Neisseria,* are common members of the human oral microbiome (Dewhirst et al., 2010). Recent molecular 16S rRNA gene studies of the oral microbiome in health, using both conventional Sanger sequencing (Aas et al., 2005, Bik et al., 2010) and high-throughput pyrosequencing (Lazarevic et al., 2009, Zaura et al., 2009), have found *Neisseria* to be one of the predominant genera. The specific species of *Neisseria* detected by Aas et al., (2005), at various sites in the oral cavity included *N. subflava*, *N. polysaccharea*, *N. bacilliformis*, *N. mucosa* and *N. elongata*. Studies have also shown that *Neisseria* spp. are among the earliest colonisers of dental plaque (Ritz, 1967, Li et al., 2004). Early colonisers can influence subsequent microbial community succession as they provide attachment sites for late colonisers via inter-species co-aggregation (Kolenbrander et al., 2006). Furthermore, as oxygen consumers, *Neisseria* species can play an important role in lowering the redox potential of developing plaque, there-by facilitating the growth of obligate anaerobes (Bradshaw et al., 1996), which can include putative periodontal pathogens. In addition, *Neisseria* species from early developing plaque have been found to produce extracellular polysaccharides (Horikawa et al., 1978), which can be metabolised by other oral bacteria. *Neisseria* are one of the common groups of oral bacteria for which 16S rRNA gene sequence-based identification is problematic, as many of the currently described and named species cannot be distinguished based on their 16S rRNA gene sequences. In such cases only an ambiguous identification with the possible alternatives can be given (Moazzez et al., 2011).

1.7.2 *Taxonomy of the genus Neisseria*

The taxonomy of the genus *Neisseria* has been complicated by the numerous changes it has undergone (Knapp, 1988), many of which resulted from the introduction of genetic taxonomic approaches, such as DNA-DNA hybridisation, DNA base composition, transformation studies and more recently 16S rRNA gene sequencing. These methods revolutionised prokaryotic taxonomy, with a movement away from the earlier emphasis on morphological traits and chemotaxonomy for classifying bacterial species. An extensive numerical phenotypic taxonomic study of the genus (Barrett and Sneath, 1994) was able to group some species into separate groups or "phenons". However, it was not possible to easily distinguish the phenotypes of the human commensal *Neisseria* species, with some different strains of the same species appearing in different phenons. The highly recombinogenic and close phylogenetic relationship of *Neisseria* species (Spratt et al., 1992, Hanage et al., 2005) further complicates the modern taxonomy of the genus by increasing the difficulties associated with accurately distinguishing different species. In addition to these issues, the on-going problems associated with clearly defining the species as a valid modern taxonomic rank is common to all bacteria and is a major subject of debate (Rossello-Mora and Amann, 2001, Cohan, 2002, Rossello-Mora, 2003, Gevers et al., 2005). The taxonomy of *Neisseria* has been the subject of previous reviews that have attempted to clarify the status of different members of the genus (Henriksen and Bovre, 1968, Henriksen, 1976, Knapp, 1988).

The genus *Neisseria,* falling within the *Neisseriaceae* family of the *Neisseriales* order of ß-Proteobacteria, includes the pathogenic species *N. meningitidis* and *N. gonorrheae*, aetiological agents of meningitis and gonorrhoea respectively. *Neisseria* can be distinguished from other genera of the family by

positive catalase and oxidase tests, their ability to reduce nitrate and to produce acid from glucose (Tønjum, 2005). The genus was named after the German bacteriologist Albert Ludwig Sigesmund Neisser, who in 1879 discovered that the sexually transmissible species *N. gonnorheae,* or *gonococcus* as it was formerly named, was the causative agent of gonnorhoea. *N. gonnorheae* remains the type strain of the genus. A number of species of *Neisseria* were described in the late 1800s and early 1900s and seven named species were subsequently listed in the first edition of Bergey's Manual of Determinative Bacteriology (Bergey et al., 1923), including several oral commensals. The online List of Prokaryotic Names with Standing in Nomenclature (LPSN) (Euzeby, 1997), currently includes 29 different species (Table 2). Of the seven species included in the first edition of Bergey's Manual, only *N. gonorroheae*, *N. sicca* and *N. subflava* are included in the LPSN. The then-named species *N. catarrhalis, N. ovis* and *N. caviae* (also known as the "false *Neisseriae"*) were moved to the genus *Branhamella* in 1974 (Reyn, 1974), following the recommendations of Henriksen and Bovre in 1967 on the basis of DNA base composition and transformation studies (Catlin and Cunningham, 1961). The genus *Branhamella* was subsequently subsumed into the genus *Moraxella* and these organisms are now known as *M. catarrhalis, M. ovis* and *M. caviae,* respectively. In addition, *N. intracellularis* was later renamed as *N. meningitidis* due to confusion arising as a result of other unrelated species from different genera using the "*intracellularis"* nomenclature (Branham and Pelczar, 1957). *N. haemolysans* was described in 1938 but was later removed from the genus and re-named *Gemella haemolysans* (Reyn, 1970). The species *N. discoides*, *N. reniformis* and *N. orbiculus* were removed from the genus in 1957 and transferred to the genus *Veillonella* (Branham and Pelczar, 1957). *Neisseria pharyngis* was proposed as a name for a

group of *Neisseria* species comprising *N. catarrhalis*, *N. flava*, *N. cinerea*, *N. mucosa* and *N. sicca,* in an attempt to simplify their taxonomy due to discrepancies in colonial morphology within strains of the same species, which at the time was a major criterion for classification of a species (Wilson and Smith, 1928). Despite still being in use, *N. pharyngis* is not a valid species name and is not listed in the LPSN.

Table 2: Members of the genus *Neisseria*

Past and present named members of the genus *Neisseria*	**Currently listed as a validly described *Neisseria* species in the LPSN**	**Listed among the species hitherto found in the human oral cavity (HOMD)**
Neisseria animalis	Yes	-
Neisseria animaloris	Yes	-
Neisseria bacilliformis	Yes	Yes
Neisseria canis	Yes	-
Neisseria catarrhalis (now *Moraxella catarrhalis*)	-	-
Neisseria caviae	Yes	-
Neisseria cinerea	Yes	-
Neisseria cuniculi	Yes	-
Neisseria denitrificans	Yes	-
Neisseria dentiae	Yes	-
Neissseria discoides	-	-
Neisseria elongata	Yes	Yes
Neisseria flava	Yes	Yes
Neisseria flavescens	Yes	Yes
Neisseria gonnorheae	Yes	-
Neisseria haemolysans (now *Gemella haemolysans*)	-	Yes
Neisseria iguana	Yes	-
Neisseria intracellularis (now *Neisseria meningitidis*)	-	-
Neisseria lactamica	Yes	-

Past and present named members of the genus *Neisseria*	**Currently listed as a validly described *Neisseria* species in the LPSN**	**Listed among the species hitherto found in the human oral cavity (HOMD)**
Neisseria macacae	Yes	-
Neisseria meningitides	Yes	Yes
Neisseria mucosa	Yes	Yes
Neisseria oralis	Yes	Yes
Neisseria orbiculata	-	-
Neisseria ovis	Yes	-
Neisseria perflava	Yes	Yes
Neisseria pharyngis	-	Yes
Neisseria polysaccharea	Yes	Yes
Neisseria reniformis	-	-
Neisseria shayeganii	Yes	-
Neisseria sicca	Yes	Yes
Neisseria subflava	Yes	Yes
Neisseria wadsworthii	Yes	-
Neisseria weaveri	Yes	Yes
Neisseria zoodegmatis	Yes	-

1.7.3 *Neisseria species found in the human oral cavity*

Of the oral *Neisseria*, *N. flava*, *N. perflava* and *N. subflava* were among the first to be described, following culture studies to determine the aetiology of meningitis (Von Lingelsheim, 1906). A subsequent meningitis study (Elser and Huntoon, 1909) described these species as Chromogenic groups I (*N. flava),* II (*N. perflava*), and III (*N. subflava*), as a result of their patterns of acid production from different carbohydrates. *N. flava* and *N. subflava* are purported to produce acid from fructose, unlike *N. perflava* which does not. In addition, *N. perflava* reportedly produces acid

and polysaccharide from sucrose where as *N*. *flava* and *N*. *subflava* do not (Tønjum, 2005). *N*. *flava* is reported to be distinguishable from *N*. *subflava* by its ability to produce acid from fructose (Knapp, 1988). There has been considerable confusion surrounding the status of these species as some later studies have suggested that they should be consolidated. Serological investigations undertaken on these species (Berger and Brunhoeber, 1961) prompted the authors to conclude that *N*. *flava* and *N*. *subflava* were similar enough to be the same species, but that they were distinct from *N*. *perflava*. The genetic transformation studies of Catlin and Cunningham (1961), however, indicated that all three were closely related enough to be considered the same species. This was subsequently proposed by Henriksen and Bovre (1968) and the names *N*. *subflava* biovar *flava*, *N*. *subflava* biovar *perflava* and *N*. *subflava* biovar *subflava* were suggested. Hence, this nomenclature has been used in a number of subsequent publications reporting on the study of these organisms (Barth et al., 2009, Bennett et al., 2012). The LPSN however, lists *N*. *flava*, *N*. *perflava* and *N*. *subflava* as separate species and maintains their original nomenclature. It therefore remains questionable as to whether or not *N*. *flava*, *N*. *perflava* and *N*. *subflava* should remain as separate valid species or be united as variants of a single species. It is interesting to note that by analysis of 16S rRNA gene sequence data through construction of a phylogenetic tree (Figure 2) from reference sequences deposited in the Human Oral Microbiome Database (Chen et al., 2010, Dewhirst et al., 2010), *N*. *flava* appears more closely related to *N*. *sicca* and *N*. *mucosa* than it does to *N*. *subflava*. Indeed, the genetic transformation studies of Catlin and Cunningham (1961) indicated that *N*. *sicca* should be included along with *N*. *flava* and *N*. *perflava* under the *N*. *subflava* name. However, the review into the taxonomy of the genus by Henriksen and Bovre (1968) recommended maintaining *N*. *sicca* as a separate

species to avoid confusion. *N. sicca* was another species first described by Von Lingelsheim (1906), and was originally given the name *Micrococcus pharyngis siccus*. The etymology of "*siccus*" being a reference to the characteristic " somewhat dry, crumbling" colonies (Bergey et al., 1923) that it forms on blood agar. *N. sicca* is reported to have the same pattern of acid production as *N. perflava* (Knapp, 1988), with acid resulting from glucose, maltose, fructose and sucrose metabolism. *Neisseria mucosa,* originally named *Diplococcus mucosus,* was also first isolated by Von Lingelsheim (1906) but was not listed among members of the *Neisseria* genus in the first edition of Bergey's Manual. The species was however, described again many years later (Véron et al., 1959) and is currently listed as a valid species in the genus by the LPSN. 16S rRNA gene sequence analysis (Figure 1) indicates that, of the validly named species, it is most closely related to *N. sicca* and *N. flava*. *N. mucosa* is reported to have an identical pattern of acid production to *N. sicca* and *N. perflava* (but not *N. flava*), however it is distinguishable by its ability to reduce nitrate (Knapp, 1988). Together, the species *N. mucosa, N. flava, N. perflava, N. subflava*, and *N. sicca*, form a group that have become referred to as the saccharolytic commensal *Neisseria* (Knapp, 1988), due to their acid-producing properties.

When a series of isolates from cases of epidemic meningitis were characterised in Chicago, USA (Branham, 1930), they were subsequently described and named as a new species: *Neisseria flavescens*, owing to the characteristic golden-yellow pigment they produced during growth. Based on 16S rRNA gene sequence analysis (Figure 1) *N. flavescens* appears to be most closely related to *N. subflava*. However, unlike the saccharolytic commensal *Neisseria* described above, *N. flavescens* reportedly does not produce acid from glucose, maltose, fructose or

sucrose (Knapp, 1988). A later extensive phenotypic taxonomic study of *Neisseria* (Barrett and Sneath, 1994), however, grouped the type strains of *N. flavescens* and *N. subflava* in the same 'phenon', with neither able to produce acids from carbohydrates, contrary to earlier reports of acid production by *N. subflava*. This led the authors to question if these taxa should be regarded as the same species under the *N. subflava* name.

Neisseria polysaccharea was described following investigation of pharyngeal meningococcal carriage among healthy children (Riou and Guibourdenche, 1987). The authors cited phenotypic characteristics, particularly the production of large amounts of extracellular polysaccharide (alluded to in the naming of the organism), as a means of distinguishing *N. polysaccharea* from its closest phylogenetic relatives (as indicated by 16S rRNA gene analysis: Figure 1) *N. meningitidis* and *N. gonorroheae,* and as grounds for its classification as a separate species.

The first rod-shaped member of the genus, subsequently named *Neisseria elongata* (Bovre and Holten, 1970) was isolated from the nasopharynx of a healthy human adult in Norway. Inclusion within the genus was justified by the authors on the basis of its ability to undergo genetic transformation of streptomycin resistance with *N. meningitidis* and *N. flava*. The species is listed in the LPSN, and has undergone division into three subspecies: *N. elongata* subsp. *elongata; N. elongata* subsp. *glycolytica;* and *N. elongata* subsp. *nitro reducens*. Based on the 16S rRNA gene tree (Figure 1) *N. elongata* appears to be more closely related to members of the genera *Eikenella* and *Kingella* than other named species of *Neisseria*. With the exceptions of the later additions *N. bacilliformis* and *N. weaveri*, *N. elongata* is unusual in the genus for being rod-shaped, however it was deemed to have shown sufficient genetic affinity to other members of the genus by transformation studies

(Bovre and Holten, 1970) to warrant inclusion. Furthermore, in common with the "true" *Neisseria*, *N*. *elongata* produces carbonic anhydrase and has a similar fatty acid composition (Tønjum, 2005). In contrast to these findings, an investigation of closely related genera using rRNA cistron similarities (Rossau et al., 1989) demonstrated that *N*. *elongata* was distinct from other members of the true *Neisseria* and formed a separate cluster with *N*. *animalis*, *N*. *canis* and *N*. *denitrificans* in the composite dendrogram constructed from the data. Another rod-shaped species *Neisseria weaveri,* was added to the genus in the 1990s (Holmes et al., 1993). Prior to being formally named, members of this species were known collectively as the Centers for Disease Control M-5 group and were isolated from infected wounds following dog bites. This species, although not having been commonly found in the human oral cavity, has been detected in a sample from a patient with refractory periodontitis (Paster et al., 2001). *Neisseria bacilliformis*, named after its bacillary morphology, was first isolated from oral cavity-related and respiratory tract infections (Han et al., 2006a). 16S rRNA gene sequencing revealed that it formed a distinct group from other members of the genus (<96%) there-by warranting classification as a separate species. The most recently described *Neisseria* species found in the human mouth is the coccus *Neisseria oralis*, formerly listed as the HOMD phylotype *Neisseria* sp. HOT014, and first isolated from the clinically healthy gingival crevice of a female subject (Wolfgang et al., 2012). The closest phylogenetic relative of *Neisseria oralis,* based on 16S rRNA phylogeny (Figure 1), appears to be an un-named *Neisseria* phylotype (HOT015).

There are currently several un-named *Neisseria* phylotypes with 16S rRNA gene sequences listed in HOMD: *Neisseria* sp. HOT015, HOT016, HOT018, HOT020, HOT499 and HOT523. The phylotypes: HOT015, HOT016, HOT018,

HOT020 were all initially identified using 16S rRNA gene clone sequences obtained in an extensive study of subgingival dental plaque (Paster et al., 2001), whilst HOT499 and HOT523 are from later unpublished data. At present, these taxa appear to be represented only by 16S rRNA gene sequences and will require isolation in culture for subsequent characterisation and naming.

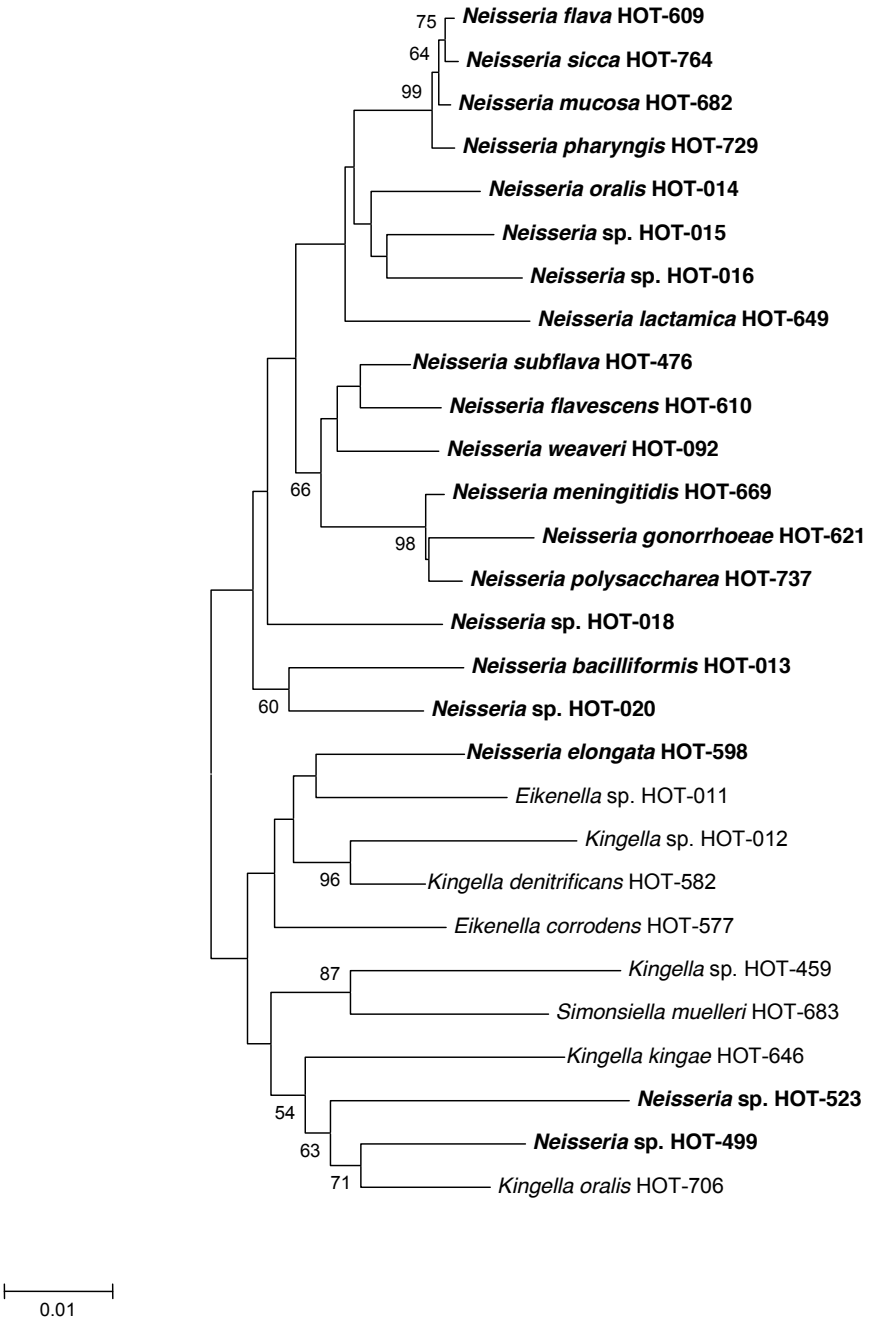

Figure 2: Phylogenetic tree of virtually full-length 16S rRNA gene reference sequences of members of the family *Neisseriaceae* downloaded from HOMD. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

1.7.4 *Molecular approaches to the identification and classification of oral Neisseria*

Contemporary approaches for the identification of *Neisseria* species in samples from the human oral cavity, as noted previously, rely heavily on the well-established 16S rRNA gene analysis. 16S rRNA genes amplified and partially sequenced with universal or specific primers can be compared to an online database of 16S rRNA gene sequences such as HOMD (Chen et al., 2010) for identification. This methodology, in practice, is limited by its resolution when attempting to identify 'fuzzy' species (Hanage et al., 2005) of a genus such as *Neisseria*. To accurately identify bacteria to the species-level a conservative 99% sequence similarity cut-off has been employed by recent studies using the 16S rRNA gene sequencing approach (Aas et al., 2005, Bik et al., 2010). It is important to note that due to the lack of clarity over the definition of a species based on 16S rRNA gene sequences alone (Fox et al., 1992, Rossello-Mora and Amann, 2001), the term species-level phylotype (Paster et al., 2001) is often used in place of species. The afore-noted significant degree of 16S rRNA gene sequence similarity and frequent horizontal gene transfer exhibited by many species of oral *Neisseria* (Spratt et al., 1992, Hanage et al., 2005), makes their accurate identification to the species-level even more challenging. The findings of a recent genome sequencing study of commensal *Neisseria* (Marri et al., 2010), further reinforced the extent to which members of the genus engage in genetic exchange, with the commensals also found to be acting as a reservoir for virulence alleles. The DNA-DNA hybridisation method in which reassociation values of lower than 70% have been used to differentiate species (Wayne et al., 1987), has been considered the gold standard for species delineation (Stackenbrandt and Ebers, 2006). DNA-DNA hybridisation is not frequently performed in most laboratories

though, as the procedure is time-consuming, labour intensive and problems arise with the influence of physico-chemical factors that can cause considerable variability in the results.

Multi-Locus Sequence Typing (MLST) is a molecular technique developed in the 1990s (Maiden et al., 1998) that can discriminate between bacteria at a high resolution, using the partial sequences of multiple 'housekeeping' genes. MLST was originally developed for *N. meningitidis*, and an ever-expanding online database of *Neisseria* MLST sequences as part of the *Neisseria* MLST scheme (PubMLST.org) is available for sequence queries (Jolley et al., 2004). Originally, MLST was designed principally for characterising populations of bacteria to the sub-species level for use in molecular epidemiological investigations. For instance, in the case of the pathogen *N. meningitidis,* MLST has proved useful in identifying the disease-causing clonal complexes, or hyper-invasive lineages (Maiden, 2006). MLST has, however, also been proposed as a tool for reliable differentiation of bacterial species (Gevers et al., 2005, Hanage et al., 2005), particularly in cases where 16S rRNA gene sequencing alone, is insufficient. Gevers et al. (2005) proposed that the term Multi-Locus Sequence Analysis (MLSA) should be used in place of MLST when applying the technique to the identification of bacteria at the species-level. Furthermore, a two-step process was considered, in which an unknown bacterium could be first assigned to a genus or family by 16S rRNA gene sequencing and subsequently primers and genes for MLSA could be chosen to allow identification to the species-level. Hanage et al. (2005) concluded that individual loci were inadequate for differentiating species of closely related *Neisseria,* where as a multi-locus approach would be considerably more effective. This was demonstrated using phylogenetic trees based on concatenated sequences of housekeeping genes that were

amplified from species of *Neisseria*. To date, no published studies have attempted to apply MLSA of the housekeeping genes used in the *Neisseria* MLST scheme to the differentiation of closely related species of oral *Neisseria*, despite the potential for it to be a useful adjunct to in-depth investigations of the oral microbiome. The MLSA approach to phylogeny and differentiation at the species-level however, has been used with some success in studies of other bacterial genera (Godoy et al., 2003, Thompson et al., 2005, Deletoile et al., 2010). Furthermore, one study has attempted to recognise the phylogenetic relationships between recombining *Neisserial* species of the human nasopharynx, by comparing sequences obtained from the housekeeping genes *argF*, *recA* and *rho* (Smith et al., 1999). The data of this study was promising, with the authors concluding that the human commensal *Neisseria* species can be separated into discrete groups of related species using such techniques, but that the relationships between these groups have been distorted by interspecies recombination events.

Another recently pioneered molecular approach for bacterial identification is that of ribosomal MLST (rMLST) (Jolley et al., 2012). This involves the use of 53 ribosomal protein subunit (*rps*) genes, which are extracted from whole-genome sequences, to construct phylogenetic trees and resolve clusters corresponding to bacterial taxa. Jolley et al. (2012) analysed the *rps* genes of bacteria from 452 genera and found that the resulting taxonomy inferred from a tree of concatenated *rps* sequences was largely consistent with that of a tree using 16S rRNA gene sequences. However, the former approach enabled higher resolution at the species-level and below, with, for example, separation of the closely related streptococcal species *S. mitis* and *S. pneumoniae*. Bennett et al. (2012) used the whole-genome sequences of 36 *Neisseria* reference strains for rMLST, as well as conventional MLST and 16S

rRNA gene analyses, in order to examine their phylogenetic relationships. The analyses demonstrated that groups largely corresponding to current species assignments could be resolved within the genus using rMLST and a minimal set of 'core' genes. However, the data also indicated that a number of *Neisseria* species deposited in culture collections have been misidentified, highlighting the problems associated with phenotypic classification of *Neisseria*. One limitation of the rMLST approach is the requirement for whole-genome sequences to extract the 53 relevant genes for analysis. Targeting of the most variable ribosomal protein gene(s) using a simple PCR and sequencing approach may be a useful alternative to rMLST.

1.8 Aims

The primary aims of this investigation were to: (i) Characterise the oral microbiota associated with periodontal health and early gingivitis; (ii) Identify candidate oral probiotics; (iii) Evaluate alternative approaches for oral *Neisseria* species differentiation.

1.8.1 *Specific aims*

- Using an 'experimental gingivitis' model and a combination of high-throughput 16S rRNA gene sequencing and conventional culture methods, determine the bacterial composition of dental plaque in periodontal health and monitor the changes that occur during the transition from health to gingivitis in the absence of oral hygiene.
- Identify specific bacterial taxa associated with periodontal health and with gingivitis.
- Investigate potentially novel oral bacterial taxa present in plaque samples of healthy individuals and chronic periodontitis patients.
- Use a 'deferred antagonism' assay to screen oral isolates recovered from healthy subjects for their antagonism towards the growth of a panel of putative periodontal pathogens, in order to determine probiotic potential.
- Develop and use a multi-locus sequence analysis scheme for phylogenetic analysis of reference strains and oral isolates of *Neisseria* species.

Chapter 2:

Analysis of the oral microbiota in experimental gingivitis

Chapter 2: Analysis of the oral microbiota in experimental gingivitis

2.1 Introduction

Gingivitis is a reversible form of periodontal disease characterised by inflammation of the gingivae in response to a mature plaque biofilm. In susceptible individuals persistent gingivitis may lead to chronic periodontitis (Schatzle et al., 2003), which causes irreversible destruction of periodontal tissue. The essential role of plaque in gingivitis was first shown through the use of an 'experimental gingivitis' model (Loe et al., 1965, Theilade et al., 1966). In this model, healthy volunteers (with minimal gingival inflammation and plaque) were instructed to abstain from all methods of oral hygiene over a period of up to three weeks. The increase in plaque led to clinically visible gingivitis in all subjects after between nine and 21 days, and was subsequently reversed when oral hygiene was reinstated and plaque removed. Using direct microscopy of the plaque samples, the investigators also noted changes in the predominant bacterial morphotypes present during the transition from health to gingivitis, findings later confirmed by culture (Loesche and Syed, 1978, Moore et al., 1982, Zee et al., 1996).

The introduction of culture-independent molecular methods to identify the bacteria present in complex samples, such as those based on cloning and Sanger sequencing of 16S ribosomal RNA genes, has greatly expanded our knowledge of oral bacterial communities (Paster et al., 2006). Studies using these techniques revealed that the oral microbiome was much more diverse than previously thought (Kroes et al., 1999, Paster et al., 2001, Kazor et al., 2003). 16S rRNA gene cloning and sequencing has provided valuable insights into differences in the composition of bacterial communities in health (Aas et al., 2005, Bik et al., 2010) and disease;

including dental caries (Becker et al., 2002, Munson et al., 2004) and chronic periodontitis (Kumar et al., 2003, Kumar et al., 2005). However, no previous studies have used 16S rRNA gene cloning and sequencing to characterise the microbiota in experimental gingivitis.

Whilst 16S rRNA gene cloning and sequencing has undoubtedly been useful, it suffers from being relatively low-throughput and researchers have typically sequenced a limited numbers of clones per sample, often approximately a hundred (Kumar et al., 2003, Aas et al., 2005). In recent years, next-generation sequencing technologies have led to significant improvements in the depth and scale of 16S rRNA sequencing studies (Rothberg and Leamon, 2008). Using 454-pyrosequencing of 16S rRNA genes, Griffen et al. (2012) and Abusleme et al. (2013) compared the subgingival bacterial communities of chronic periodontitis patients to those of healthy individuals. In both cases the authors elucidated significant differences between the cohorts in terms of the membership and structure of their subgingival communities and reported higher bacterial diversity in disease. In addition, the authors revealed associations between specific bacterial taxa and disease. The 'red complex' species, first described by Socransky et al. (1998): *P. gingivalis*, *T. denticola* and *T. forsythia*, were associated with disease in both studies. Interestingly, however, they also identified a range of additional species that showed associations with chronic periodontitis *F. alocis* and *Treponema medium* were among those taxa most strongly associated with disease in both studies. Huang et al. (2011) used 454-pyrosequencing to explore bacterial community differences in healthy individuals and those with gingivitis in samples of saliva and supragingival plaque. The communities in plaque, but not in saliva, differed significantly according to health or gingivitis. Indeed, a number of species-level OTUs in dental plaque were enriched or

reduced in the individuals with gingivitis versus healthy individuals. Many of the OTUs associated with gingivitis were identified as members of the genera *Leptotrichia* and *Selenomonas*, although species-level assignments were not provided in most cases. One limitation of this and other cross-sectional studies though, is that large inter-individual differences, particularly at the species-level, have been found in the oral microbiome (Kroes et al., 1999, Diaz et al., 2006, Diaz et al., 2012). This variation suggests that it may be difficult to determine a shared 'core' microbiome in health with which the microbiome found in individuals with a particular state of disease can be compared.

This work described in this chapter aimed to combine the experimental gingivitis model with high-throughput 16S rRNA gene pyrosequencing and culture to comprehensively characterise the bacterial communities in plaque during the transition from health to experimentally-induced gingivitis. In addition, plaque samples from a group of patients with severe periodontitis were pyrosequenced as a cross-sectional comparison to the healthy cohort. The analyses sought to compare the bacterial communities found in periodontal health, the initial stages of gingivitis and chronic periodontitis and to identify bacterial taxa associated with each health and disease state.

2.2 Methods

2.2.1 *Study design*

The study was comprised of both cross-sectional and longitudinal experimental phases. A pre-experimental phase was carried out during which suitable clinically healthy volunteers were recruited as well as patients with severe chronic

periodontitis. A total of 40 participants were recruited: 20 healthy volunteers and 20 with chronic periodontitis.

2.2.2 *Subject recruitment*

Ethical approval for the study was granted by the South East London Research Ethics Committee 1 (formerly Guy's REC) and informed consent was obtained from all individuals who participated. All patients and subjects enrolled in the study had at least 20 teeth and were systemically healthy with no history of antibiotic use for at least three months prior to the study. Pregnant women, current smokers or individuals who quit smoking within the previous five years were not enrolled.

2.2.2.1 Periodontally healthy volunteers

Recruitment was made from clinical staff within the King's College London Dental Institute. All subjects recruited had no evidence of periodontitis and had minimal gingival inflammation. The BPE (Basic Periodontal Examination) was used to screen potential subjects. Subjects had no BPE score greater than one in any of the sextants, no probing attachment loss and no evidence of gingival recession. Gingival bleeding was used to assess gingival inflammation and subjects were only enrolled if fewer than 15% of sites bled after probing. All clinical examinations were undertaken by the same dentally-qualified clinician.

2.2.2.2 Patients with chronic periodontitis

Patients with chronic periodontitis were enrolled from those referred to the Department of Periodontology at Guy's and St Thomas' Foundation Trust for treatment. All patients had a minimum of 20 teeth and were diagnosed with severe chronic periodontitis (Armitage, 1996). Each patient had at least six teeth with probing depths of ≥6mm and bone loss.

2.2.3 *Experimental gingivitis*

A summary of the experimental gingivitis study design is shown in Figure 3. On the morning of the baseline appointment, subjects were instructed to brush their teeth as normal and appointments were scheduled for approximately four hours after brushing. Following the baseline examination, subjects were instructed to abstain from all methods of tooth cleaning (including brushing, interdental cleaning and mouth rinsing) in the mandible for two weeks. A soft acrylic stent was made to cover the mandibular teeth whilst brushing the maxillary teeth and was removed immediately after brushing. The soft stent could be easily inserted and removed without disturbance of the developing plaque whilst ensuring subjects could not brush their mandibular teeth and thus disrupt plaque formation. All subjects were reviewed after one and two weeks of plaque accumulation. At baseline and after two weeks six sites/tooth were probed and bleeding on probing (BoP) was assessed. Probing after one week of plaque accumulation was avoided since bleeding in the crevice might have influenced the developing biofilm. However, at all time points non-invasive samples of gingival crevicular fluid (GCF) were collected before clinical measurements or plaque sampling. The number of sites with clearly visible plaque (Silness and Loe plaque index=2 (Silness and Loe, 1964)) were recorded. After two weeks all subjects had their teeth polished and they resumed normal oral hygiene practices.

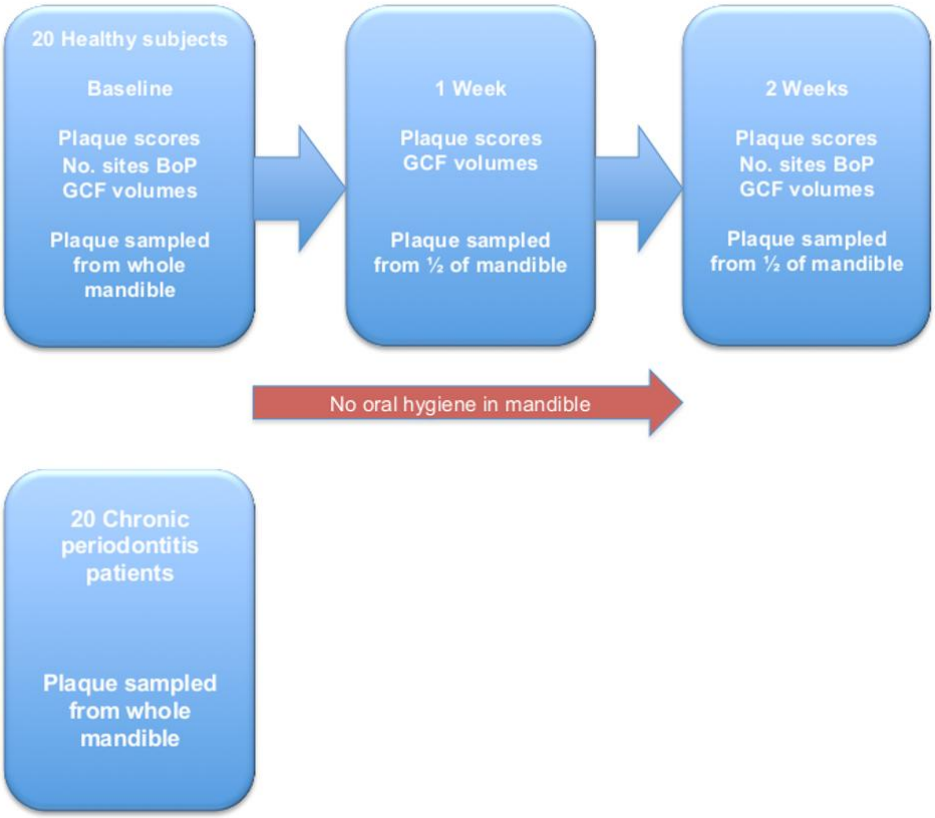

Figure 3: Study design.

2.2.4 *Sample collection*

2.2.4.1 Experimental gingivitis

At baseline, after collection of GCF and clinical measurements, plaque samples were collected using a sterile curette from all the mandibular teeth in the healthy subjects with the exception of the third molars. Plaque samples were collected using a sterile curette from just above the gingival margin and from the gingival crevices. Plaque from all sites within the subject was pooled and immersed in 1 ml of sterile 0.1x Tris-EDTA, and processed immediately. GCF was collected from the mesiobuccal sites of 12 mandibular teeth on periopaper strips and the volume of fluid estimated using a Periotron 8000. The strips were stored in Phosphate Buffered

Saline (PBS) at -70°C. Samples of plaque and GCF were collected again after one and two weeks of plaque accumulation. Plaque was collected from teeth on one half of the mandible after one week and the other half after two weeks.

2.2.4.2 Chronic periodontitis

Superficial plaque samples were collected in the same way as those from the healthy subjects. For 14 of the 20 patients, separate samples of subgingival plaque were collected by inserting a curette to the full depth of pockets $\geq$ 6 mm, after the superficial plaque had been collected. These sites were selected on the basis of pocket depth $\geq$ 6 mm and the specific sites selected varied between individuals depending on the pattern of their disease. The clinical condition of the patients was so different from the group of healthy volunteers it was impossible to blind the examiner to which group the individuals came from. However, the use of a sampling technique that removed plaque from around the gingival margins and to the depth of a healthy crevice in both the experimental gingivitis and periodontitis groups standardised the physical environment from which the superficial plaque samples were collected. The superficial plaque from the healthy volunteers and periodontitis patients was used for inter-group comparison and an additional comparison was made between the superficial and subgingival samples within the patients.

2.2.5 ***DNA extraction***

DNA was extracted from the samples using the GenElute Bacterial DNA Extraction Kit (Sigma-Aldrich). Extractions were carried out following the manufacturer's instructions with an additional lysis step to increase the recovery of Gram-positive bacterial DNA: samples were incubated with a 45 mg/ml lysozyme solution at 37°C for 30 minutes. Extracted DNA was stored at -70°C until further processing.

2.2.6 *16S rRNA gene PCR and 454 pyrosequencing*

An approximately 500 bp region of the 16S rRNA gene (covering hyper variable regions 1-3) was PCR-amplified from extracted DNA samples using composite fusion primers comprising the previously described 'universal' 16S rRNA gene primers: 27FYM (Frank et al., 2008) and 519R (Lane et al., 1985), along with Roche GS-FLX Titanium Series adapter sequences (A & B) for 454 pyrosequencing using the Lib-L emulsion-PCR (emPCR) method. Previously described unique 12 base error-correcting Golay barcode sequences (Fierer et al., 2008) were incorporated into the forward primers (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-NNNNNNNNNNNN-AGAGTTTGATYMTGGCTCAG-3') to enable pooling of samples in the same sequencing run. The full list of molecular barcodes used is shown in Appendix A. PCR was performed using the appropriate A-27FYM and B-519R (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-GWATTACCGCGGCKGCTG-3') primers and Extensor Hi-Fidelity PCR Master Mix (Thermo-scientific) in a final reaction volume of 25 µl: 12.5 µl of Master Mix; 9.5 µl of sterile molecular grade H_20 (Sigma); 0.5 µl each of 10 pmol/µl forward (A-27FYM) and 10 pmol/µl reverse primer (B-519R); and 2 µl DNA template. The PCR reaction conditions were as follows: 5 minutes denaturation at 95°C followed by 25 cycles of 95°C for 45 s, 53°C for 45 s, 72°C for 1 min 30 s and a final extension of 72°C for 15 minutes. A negative control reaction (no template) was included for every PCR performed and each primer set. PCR amplicons were then visualised using gel electrophoresis on a 1% agarose gel (stained with GelRed™, Biotium) under UV light, to ensure the presence of amplicons of approximately the correct size (~560 bp). Amplicons were then purified using the QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions, and eluted into 30 µl of

sterile 0.1x Tris-EDTA. The purity and size of the amplicons was checked by electrophoresis in microfluidic chips (DNA 1000 kit) on the 2100 Bioanalyzer (Agilent Technologies). The concentrations of the amplicons were then determined by fluorometry using the Picogreen dsDNA quantification assay kit (Invitrogen), according to the manufacturer's instructions, and pooled together in equimolar concentrations (1 x 10^9 molecules/µl). emPCR and unidirectional sequencing of the libraries was performed using the Lib-L kit, using a Roche 454 GS-FLX Titanium sequencer at the Centre for Haemato-Oncology, Bart's Cancer Institute, Queen Mary University of London, London, UK.

2.2.7 *Sequence analysis*

Pre-processing and analysis of sequences was carried out using the mothur analysis suite version 1.26.0 (Schloss et al., 2009) based on the Schloss standardised operating procedure (SOP) (Schloss et al., 2011). Sequences were initially de-noised using the AmpliconNoise algorithm, as implemented by mothur (Quince et al., 2011). After de-noising, any sequences that were less than 350 bp in length and/or had one of the following: >2 mismatches in the primer, >1 mismatch in barcode regions and homopolymers of >8 bases, were removed from the dataset. The remaining sequences were trimmed to remove the primers and barcodes and then aligned to the SILVA 16S rRNA reference alignment (Pruesse et al., 2007). The UChime algorithm (Edgar et al., 2011) as implemented by mothur was used to identify chimeric sequences, which were removed from the dataset. A phylogenetic neighbour-joining tree of all sequences was constructed using Clearcut (Evans et al., 2006), as implemented by mothur. Sequences were identified by BLAST against the Human Oral Microbiome Database (HOMD) (Chen et al., 2010) at ≥ 98.5% sequence identity. Sequences were also clustered into Operational Taxonomic Units

(OTUs) at a sequence dissimilarity distance of 0.015 using an average neighbour algorithm and then classified using a Naïve Bayesian classifier implemented in mothur with the HOMD version 10.1 reference dataset. Where species-level assignment of a particular OTU was not possible using the Bayesian classifier, BLAST analysis in HOMD was performed (as described above), and the possible alternatives for the species identification were given. A previously described method was used to distinguish the commensal mitis-group streptococci from the closely related pathogen *Streptococcus pneumoniae* (Scholz et al., 2012). The α-diversity of bacterial communities based on OTUs was analysed using a number of approaches implemented by mothur: Good's non-parametric coverage estimator (Good, 1953) was used to assess the extent of sampling of the communities. Diversity of the communities was calculated using Simpson's inverse diversity index (Simpson, 1949) and the total richness of the communities was estimated using Chao1 (Chao, 1984) and CatchAll (Bunge, 2011). For the β diversity analyses the dataset was sub-sampled so that the number of sequences were equal in all samples (equal to that of the sample with the lowest number of sequences in the dataset). The Jaccard Index (ratio of shared OTUs to distinct OTUs) and the thetaYC metric (compares community structure by accounting for the relative abundance of taxa) (Yue and Clayton, 2005) were used to generate distance matrices from sub-sampled sequence libraries (equal to that of the library with the fewest sequences), which were visualised as dendrograms and Principal Coordinates Analysis (PCoA) plots. Three-dimensional PCoA plots were generated in R (r-project.org) using the rgl package. The β diversity of communities was also compared based on their phylogenetic relatedness. For this, a neighbour-joining tree of the sequences constructed with Clearcut (Evans et al., 2006) was analysed by unweighted and weighted UniFrac

metrics (Lozupone and Knight, 2008) as implemented by mothur, and visualised as above.

2.2.8 *Statistical analysis*

The non-parametric Friedman and Wilcoxon-signed rank tests were used to test for the significance of differences in the OTU richness and diversity of samples from the different time points of experimental gingivitis. A Bonferroni correction for multiple comparisons was applied to the alpha value for pairwise comparisons. Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) was performed in mothur to determine if clustering patterns seen in the PCoA plots were statistically supported by differences in the distance matrices. A Bonferroni correction for multiple comparisons was applied to the alpha value when comparing the time points of experimental gingivitis. Parsimony (Slatkin and Maddison, 1990), as implemented by mothur, was used to determine if clustering in the dendrograms was significant. Associations of OTUs with time points of experimental gingivitis and BoP scores were detected using Multivariate Association with Linear Models (MaAsLin) (Morgan et al., 2012). Linear Discriminant Analysis Effect Size (LEfSe) (Segata et al., 2011) was used to detect differentially abundant OTUs between healthy and chronic periodontitis cohorts. The alpha values in LEfSe were set to 0.05 and an LDA threshold of 2.0 was applied. Two-sample *t*-tests and paired *t*-tests were performed in R to determine if differences in the relative abundances of phyla between the healthy and periodontitis cohort and between time points of experimental gingivitis were statistically significant. A Bonferroni correction for multiple comparisons was applied to the alpha values. Dichotomous plaque and bleeding scores were expressed as a percentage of the number of assessed sites. Percentage plaque scores were first analysed using the Friedman test for non-

parametric data to establish whether there was a statistically significant difference in the amount of plaque over the three time points and this was followed by comparison between time points using Wilcoxon sign-rank tests. Since percentage bleeding scores were only measured at two time points they were analysed using a Wilcoxon sign-rank test. The volume of GCF was a continuous, normally distributed variable and was therefore first analysed using a repeated measures analysis of variance and subsequently comparison between individual time points assessed using paired t-tests.

2.2.9 *Bacterial culture analysis*

For ten of the healthy subjects, baseline and two week plaque samples were cultured on non-selective media. First, 500 µl of a 0.1x Tris-EDTA suspension containing plaque was sonicated for 20 s. Ten-fold serial dilutions of the sonicated samples (up to 10^5) were made in pre-reduced transport medium and inoculated onto Blood Agar Base no. 2 + 5% v/v horse blood (BA) and Fastidious Anaerobe Agar + 5% horse blood (FAA), in triplicate. BA plates were incubated for 4 days in 5% $C0_2$ + air at 37°C, whilst FAA plates were incubated for 10 days in an anaerobic cabinet with an atmosphere of 80% N_2, 10% H_2 and 10% $C0_2$ at 37°C. After incubation, plates were counted and subculture of colonies was performed from a suitable dilution. Random selection of colonies was ensured as previously described (de Lillo et al., 2006) and 96 colonies were sub-cultured for each sample (48 from BA and 48 from FAA). Once pure cultures were obtained, 16S rRNA genes were amplified from the isolates as follows: A 'touch' PCR method was used whereby a sterile pipette tip was gently touched onto a single colony of the culture and the cell material was suspended into 50 µl of sterile molecular grade water (Sigma). 1 µl of this suspension was used in a PCR reaction mix comprised of 23 µl of Thermoprime PCR Master Mix (ABgene,

UK), 0.5 µl each of the forward and reverse 'universal' primers 27FYM and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') at a concentration of 10 pmol/µl, for a final volume of 25 µl. The PCR reaction conditions were: 5 minutes denaturation at 95°C followed by 30 cycles of 95°C for 45 s, 56°C for 45 s, 72°C for 1m 30s and a final extension of 72°C for 15 min. A negative control reaction (no template added) was included for every PCR performed. PCR amplicons were then visualised using gel electrophoresis on a 1% agarose gel (stained with GelRed™, Biotium) under UV light, to ensure the presence of amplicons of the correct size (~1500 bp). Next, un-used primers and nucleotides were removed from the PCR products using Exo-SAP-IT (USB) following the manufacturer's instructions with the following modified reaction recipe: 5 µl of PCR product, 1 µl of sterile molecular grade water (Sigma), and 1 µl of Exo-SAP-IT enzyme. The purified 16S rRNA gene amplicons were partially sequenced using primer 519R and the BigDye® Terminator version 3.1 cycle sequencing kit (Invitrogen) on a thermal cycler with the following reaction recipe: 0.5 µl of BigDye®, 1.75 µl of 5x sequencing buffer (400 mM Tris Base pH 9.0, 10 mM $MgCl_2$), 5.45 µl of sterile molecular grade water (Sigma), 0.3 µl of 10 µM primer, and 2 µl of purified PCR product. The sequencing reaction conditions were as follows: 30 cycles of 10 s at 96°C, 5 s at 50°C, and 2 min at 60°C. Sequencing products were cleaned using ethanol and sodium acetate precipitation, separated by capillary electrophoresis and read on the ABI 3730xl DNA analyser (Applied Biosystems). Sequence chromatograms were inspected in the ABI sequence scanner software and subsequently exported in the FASTA format for further analysis. Sequences were edited and aligned (using ClustalW) in the BioEdit sequence alignment editor. The sequences were then assigned identities by BLAST search against HOMD (≥98.5% sequence identity). In addition, sequences were grouped by

sample and incubation condition and analysed in the mothur software suite as follows: Sequences were first aligned to the SILVA 16S rRNA reference set. The alignment was then used to generate a distance matrix and sequences were clustered into OTUs using the average neighbour algorithm at a distance of 0.015. The α-diversity of samples was assessed using Good's coverage, Simpson's inverse diversity index and Chao1 calculators as implemented by mothur.

2.2.10 *Investigation of potentially novel oral taxa*

Phylotypes that did not match reference taxa in HOMD (<98.5% sequence identity) and were present in at least two individuals were investigated further. Specific forward primers were designed with reference to the sequences obtained by 454-pyrosequencing and used to amplify the 16S rRNA gene of the target phylotype, in combination with the 'universal' primer 1492R, from the extracted DNA samples. Primers were designed to be identical to the target sequence and to have a minimum of two mismatches to taxa in the HOMD v10.1 reference dataset. PCRs were performed as described in the previous section, but using the specific forward primers (list of primers used is shown in Table 3) in combination with the universal reverse primer 1492R. PCR amplicons of the correct size (~1400-1500 bp depending on the forward primer used) were subsequently cloned using the TOPO TA Cloning® kit (Invitrogen, UK) with PCR® 4-TOPO® plasmid vector and One Shot® TOP10 competent *E. coli* cells, according to the manufacturer's instructions. *E. coli* transformants were grown overnight on Luria Bertani agar supplemented with 50 μg/ml of kanamycin sulphate. The 16S rRNA inserts were amplified using the 'touch PCR' protocol described earlier and M13 primers: M13(-20)F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') targeting the TOPO plasmid vector, with the following amplification protocol: initial

denaturation at 95˚C for 15 minutes followed by 30 cycles of 95˚C for 45 s, 55˚C for 45 s and 72˚C for 90 s. Clones were sequenced using the BigDye® Terminator v3.1 cycle sequencing kit, as described in the previous section. The previously described universal 16S rRNA gene sequencing primers 342R (5'-CTGCTGCSYCCCGTAG-3'), 357F (5'-CTCCTACGGGAGGCAGCAG-3'), 519R, 907R (5'-CCGTCAATTCCTTTRAGTTT-3'), 926F (5'-GGTTAAAACTYAAAKGAATTGACGG-3'), 1100R (5'-GGGTTGCGCTCGTTG-3') 1114F (5'-GCAACGAGCGCAACCC-3'), 1392R (5'-ACGGGCGGTGTGTRC-3'), and 1492R, were used to obtain virtually full-length sequences for clones that matched the desired target upon visual inspection of an alignment to the 454 sequences in the BioEdit software. Additionally, virtually full-length sequencing of potentially novel isolates (<98.5% identity to taxa in HOMD) from the culture analysis was performed using the same set of universal primers.

Table 3: Specific primers designed and used for PCR and sequencing of novel phylotypes. Primes are named according to the taxa targeted and the numbering indicates the position of the primer with respect to the *E. coli* 16S rRNA gene (determined by visual inspection of sequence alignments).

Primer name	**Sequence 5'-3'**
Molli-93-F	AACGGAGTCTTTTAGAC
Actino-71-F	CCTGCTCCTTGTGGGTG
Allo-133-F	TATCGAACCTACCACATAC
Lept-74-F	ACATGCAAGTCTTTGGCGAACC
Berg-96-F	ATTGGTTAGCTTGCTAACTGAG
Tane-154-F	GCCCGCAACAGAGGGATAATTC
Propi-96-F	TCCTTCGGGAGTACACGAGTG

2.3 Results

2.3.1 *Clinical results*

The experimental gingivitis cohort comprised 16 females and four males with a mean age of 28.1 (± 2.1) years. 19 of the subjects completed the study. One subject withdrew from the study because of a chest infection. No other adverse affects or complications were reported. After two weeks all subjects had developed thick, clearly visible plaque on most tooth surfaces. They had significantly increased gingival bleeding on probing (BoP) scores (Table 4) and the mean volume of gingival crevicular fluid (GCF) increased significantly after one and two weeks. A total of 11 female and nine male chronic periodontitis patients with a mean age of 48.5 (± 9) years were sampled. A two-sample *t*-test showed that periodontitis patients were significantly older than the experimental gingivitis group. The mean number of teeth per patient was 28.1 (±2.8) whilst the number of teeth per patient with pocket depths ≥6 mm was 11.9 (±5.9). In 14 of the patients, subgingival plaque samples from deep pockets were obtained as an additional comparison to the plaque samples from around the gingival margin.

Table 4: Summary of clinical parameters of subjects during experimental gingivitis.

Time point	**GCF volume (µl)** **Mean (±SD)**	**% bleeding sites** **Median (IQR)**	**% of sites with visible plaque Median (IQR)**
Baseline	6.82 (±2.01)	7.04 (4.8-10.7)	6.7 (1.8-10.0)
1 Week	8.22 (±1.94)	N/A	62.4 (50.1-79.5)
2 Weeks	9.77(±2.21)	37.2 (29.8-47.2)	87.02 (71.1-92.9)
	ANOVA: $P<0.00001$ Paired t-tests indicated significant differences between all time points ($P<0.019$).	Wilcoxon signed-rank test: $P<0.00001$.	Friedman test $P<0.00001$ Wilcoxon signed-rank test indicated significant differences between all time points ($P<0.0001$).

2.3.2 *Pyrosequencing summary*

394,558 sequences with a mean length of 423 bases were obtained after initial quality filtering. Alignment to the SILVA reference database, subsequent screening of the alignment and removal of chimeric sequences resulted in a final dataset of 344,267 high quality sequences (of which 31,604 were unique) with a mean length of 354 bases. This provided a final mean yield of 3742 (ranging between 2156 and 5811) sequences per sample for further analysis.

2.3.3 *OTU-based alpha and beta diversity of bacterial communities in plaque*

Clustering of sequences into OTUs at a distance of 0.015 resulted in 89 to 394 (median 299) species-level OTUs per plaque sample/bacterial community. Mean coverage of the communities according to Good's non-parametric calculator was 96.9% (±0.7%). Chao1 estimates of total OTU richness ranged from 130 to 634 OTUs (median 464), whilst CatchAll gave estimates from 200 to 1690 (median 644). The observed OTU richness and diversity of communities (Simpson's inverse

diversity index) at different sampling times of experimental gingivitis are shown in Figure 4. The richness of the communities across time points was significantly different (Friedman test, $P<0.016$). Using pairwise Wilcoxon signed-rank tests there was no significant difference between baseline communities and the one- and two-week communities. However, the number of OTUs was significantly higher in two-week communities compared to one-week communities ($P<0.01$). Because the number of observed OTUs changes with sampling depth, communities were sub-sampled to compare the richness across samples with the same number of sequences (2156). The analysis gave the same results. The diversity of the communities was assessed using Simpson's inverse diversity index which takes into account both richness and evenness. Collector's curves for all samples (not shown) indicated that diversity estimates using this index remained stable at differing sequencing depths above approximately 500 sequences, thereby allowing comparison of diversity across samples with different sampling depths. There was a significant difference in diversity across time points (Friedman test, $P<0.00044$). Pairwise Wilcoxon-signed rank tests showed that diversity was significantly higher in two-week communities compared to both baseline ($P<0.0001$) and one-week communities ($P<0.0012$), but that there was no significant difference between baseline and one-week communities. The alpha-diversity parameters for each of the 92 plaque samples are summarised in Table 5. Only eight OTUs, assigned to the taxa: *Actinobaculum* sp. HOT183, *Campylobacter gracilis*, *Campylobacter showae*, *Cardiobacterium hominis*, *Fusobacterium nucleatum* subsp. *polymorphum*, *Lautropia mirabilis*, *Streptococcus mitis*/ HOT064/HOT423/HOTA95/HOTE14 and *Veillonella parvula*, were detected in all healthy subjects' baseline samples. Three OTUs, assigned to the taxa *Fusobacterium nucleatum* subsp. *animalis*, *Fusobacterium nucleatum* subsp.

vincentii, and *Streptococcus mitis*/ HOT064/HOT423/HOTA95/HOTE14 were shared among all of the periodontitis patients.

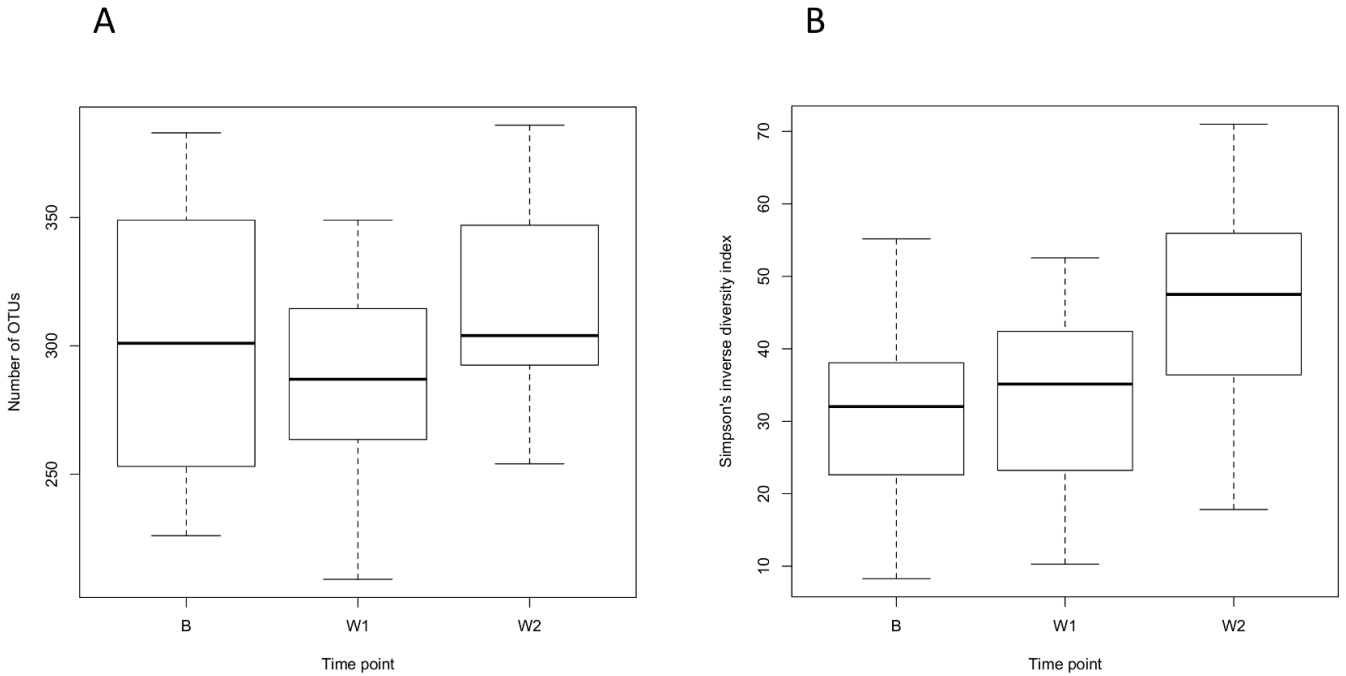

Figure 4: Richness and diversity of plaque during the induction of experimental gingivitis. Box-and-whisker plots comparing the species-level OTU richness and diversity of time points during experimental gingivitis. The top and bottom boundaries of the boxes show the 75th and 25th percentile and the ends of the whiskers show the maximum and minimum values. Bold lines within the boxes represent median values (50th percentile). (A) Number of observed OTUs at baseline (B), one week (W1) and two weeks (W2). (B) Simpson's inverse diversity index at baseline (B), one week (W1) and two weeks (W2).

Table 5: Alpha diversity of dental plaque samples. HS=healthy subject, B=baseline, 1W=one week, 2W=two weeks, CP= chronic periodontitis, Sub=subgingival. Numbers indicate subject number.

Sample ID	No. of seqs	No. of observed OTUs	Good's coverage (%)	Chao1 total OTU richness estimate	CatchAll total OTU richness estimate	Simpson's inverse diversity index	Simpson's evenness
HS1_B	3061	326	95.6	508	738	37.7	0.12
HS1_1W	4663	349	97.2	535	1034	46.1	0.13
HS1_2W	3303	283	97.1	455	881	48.6	0.17
HS2_B	3479	201	97.2	434	435	14.5	0.07
HS3_B	4722	243	97.4	543	780	8.3	0.03
HS3_1W	4526	332	97.1	530	926	30.9	0.09
HS3_2W	5104	361	97.4	529	838	40.8	0.11
HS4_B	4978	301	97.4	509	751	17.3	0.06
HS41_W	5542	299	98.0	457	1690	22.6	0.08
HS4_2W	5271	350	97.8	469	659	39.4	0.11
HS5_B	3210	316	96.0	502	688	32.5	0.10
HS5_1W	3464	281	96.4	484	610	37.8	0.13
HS5_2W	3075	327	95.9	491	676	43.9	0.13
HS6_B	3176	250	96.7	384	547	18.9	0.08
HS6_1W	2697	258	96.2	442	876	42.7	0.17
HS6_2W	3559	329	96.9	478	689	71.2	0.22
HS7_B	3151	226	96.7	388	612	22.2	0.10
HS7_1W	2698	253	96.3	374	638	33.3	0.13
HS7_2W	3603	300	97.2	407	541	48.4	0.16
HS8_B	3549	256	96.7	478	474	25.2	0.10
HS8_1W	4439	268	97.5	466	499	11.6	0.04
HS8_2W	2941	287	96.4	431	470	47.9	0.17
HS9_B	3220	354	95.7	520	804	46.1	0.13
HS9_1W	3231	311	96.3	446	595	37.3	0.12
HS9_2W	3292	325	96.8	419	549	59.2	0.18
HS10_B	3698	369	96.1	570	817	52.7	0.14
HS10_1W	3135	339	95.5	586	884	50.3	0.15

Sample ID	No. of seqs	No. of observed OTUs	Good's coverage (%)	Chao1 total OTU richness estimate	CatchAll total OTU richness estimate	Simpson's inverse diversity index	Simpson's evenness
HS10_2W	3573	386	96.1	557	668	64.5	0.17
HS11_B	3294	297	96.6	483	603	44.3	0.15
HS11_1W	3313	344	95.9	544	978	49.2	0.14
HS11_2W	2909	298	96.3	433	675	46.1	0.15
HS12_B	4121	376	96.2	577	807	30.3	0.08
HS12_1W	3276	312	96.0	469	725	17.4	0.06
HS12_2W	2876	302	96.0	445	507	34.2	0.11
HS13_B	3536	280	96.6	487	550	23.3	0.08
HS13_1W	3106	250	96.6	392	536	24.8	0.10
HS13_2W	4159	303	97.1	482	1161	18.1	0.06
HS14_B	4162	344	96.4	592	764	36.6	0.11
HS14_1W	3487	271	97.2	412	776	37.3	0.14
HS14_2W	3606	254	97.4	397	438	35.1	0.14
HS15_B	4350	380	96.8	557	649	45.7	0.12
HS15_1W	3550	317	96.7	443	585	48.3	0.15
HS15_2W	3997	385	96.4	576	730	59.4	0.15
HS16_B	4343	302	97.3	510	531	38.0	0.13
HS16_1W	5746	290	97.9	532	563	10.2	0.04
HS16_2W	4469	346	97.2	498	668	49.7	0.14
HS17_B	4049	246	97.6	369	468	16.7	0.07
HS17_1W	3357	209	97.9	293	522	31.1	0.15
HS17_2W	4141	261	97.6	426	459	52.7	0.20
HS18_B	3363	249	97.0	423	522	23.4	0.09
HS18_1W	2973	259	96.6	406	553	41.5	0.16
HS18_2W	3545	273	96.5	491	866	27.0	0.10
HS19_B	5811	383	97.4	625	834	32.2	0.08
HS19_1W	3885	287	97.2	437	591	34.6	0.12
HS19_2W	3896	348	97.0	496	525	63.5	0.18
HS20_B	3732	262	97.2	433	817	32.9	0.13
HS20_1W	3956	281	97.1	448	829	20.2	0.07

Sample ID	No. of seqs	No. of observed OTUs	Good's coverage (%)	Chao1 total OTU richness estimate	CatchAll total OTU richness estimate	Simpson's inverse diversity index	Simpson's evenness
HS20_2W	4294	304	97.2	461	731	33.0	0.11
CP1	3803	358	95.8	603	928	21.3	0.06
CP2	3128	373	95.4	586	977	71.7	0.19
CP3	4001	330	96.6	554	1021	47.6	0.14
CP4	3536	294	97.0	418	530	29.9	0.10
CP5	4289	386	96.5	581	1006	54.1	0.14
CP6	3494	394	95.9	557	821	64.2	0.16
CP7	3472	367	96.0	583	743	47.0	0.13
CP7_Sub	3839	309	97.3	418	631	50.9	0.16
CP8	5797	308	97.7	503	869	14.8	0.05
CP8_Sub	3842	268	97.1	438	1059	11.5	0.04
CP9	3801	287	96.7	516	620	24.7	0.09
CP9_Sub	4492	350	96.9	541	653	42.0	0.12
CP10	3288	330	95.9	537	830	45.0	0.14
CP10_Sub	4859	394	96.8	634	858	56.4	0.14
CP11	5304	241	98.4	345	585	17.9	0.07
CP11_Sub	4405	250	98.1	356	349	29.0	0.12
CP12	5201	355	97.0	609	1204	23.8	0.07
CP12_Sub	4782	345	97.3	516	566	13.4	0.04
CP13	2478	89	98.7	130	200	13.2	0.15
CP13_Sub	2810	176	97.9	269	322	15.8	0.09
CP14	2156	136	97.5	225	298	12.9	0.09
CP14_Sub	3017	186	97.7	290	290	18.5	0.10
CP15	2813	260	96.4	375	599	32.5	0.12
CP15_Sub	2847	271	96.8	366	505	48.6	0.18
CP16	3956	211	98.2	357	390	22.8	0.11
CP16_Sub	3657	201	98.1	325	1152	15.3	0.08
CP17	2651	216	97.7	266	350	26.8	0.12
CP17_Sub	3112	158	98.4	203	240	9.2	0.06
CP18	3899	331	97.3	455	637	39.3	0.12

Sample ID	No. of seqs	No. of observed OTUs	Good's coverage (%)	Chao1 total OTU richness estimate	CatchAll total OTU richness estimate	Simpson's inverse diversity index	Simpson's evenness
CP18_Sub	3113	301	96.9	400	573	51.1	0.17
CP19	2875	235	96.4	478	1039	41.3	0.18
CP19_Sub	3310	250	97.4	371	435	36.9	0.15
CP20	2856	231	97.1	360	431	42.6	0.18
CP20_Sub	3722	242	97.4	376	558	32.1	0.13

Comparisons of the bacterial community membership (Jaccard index and unweighted UniFrac) of the plaque samples using dendrograms, revealed that communities from the different time points of experimental gingivitis clustered principally by subject, as shown in Figure 5. A separate comparison of baseline plaque communities from the healthy cohort to superficial plaque from chronic periodontitis patients showed some clustering by cohort (Figure 6). Analysis of superficial and subgingival plaque samples from periodontitis patients showed that communities clustered by patient rather than by the type of plaque sample (Figure 7). A parsimony analysis was conducted for each dendrogram to assess the significance of clustering patterns of groups and time points. There was a significant difference between the clustering of the baseline communities of healthy subjects and superficial plaque communities of periodontitis patients ($P<0.036$ and $P<0.012$ for Jaccard index and unweighted UniFrac dendrograms, respectively). There were no significant differences between the clustering of communities from different time points of experimental gingivitis or between superficial and subgingival plaque communities from periodontitis patients.

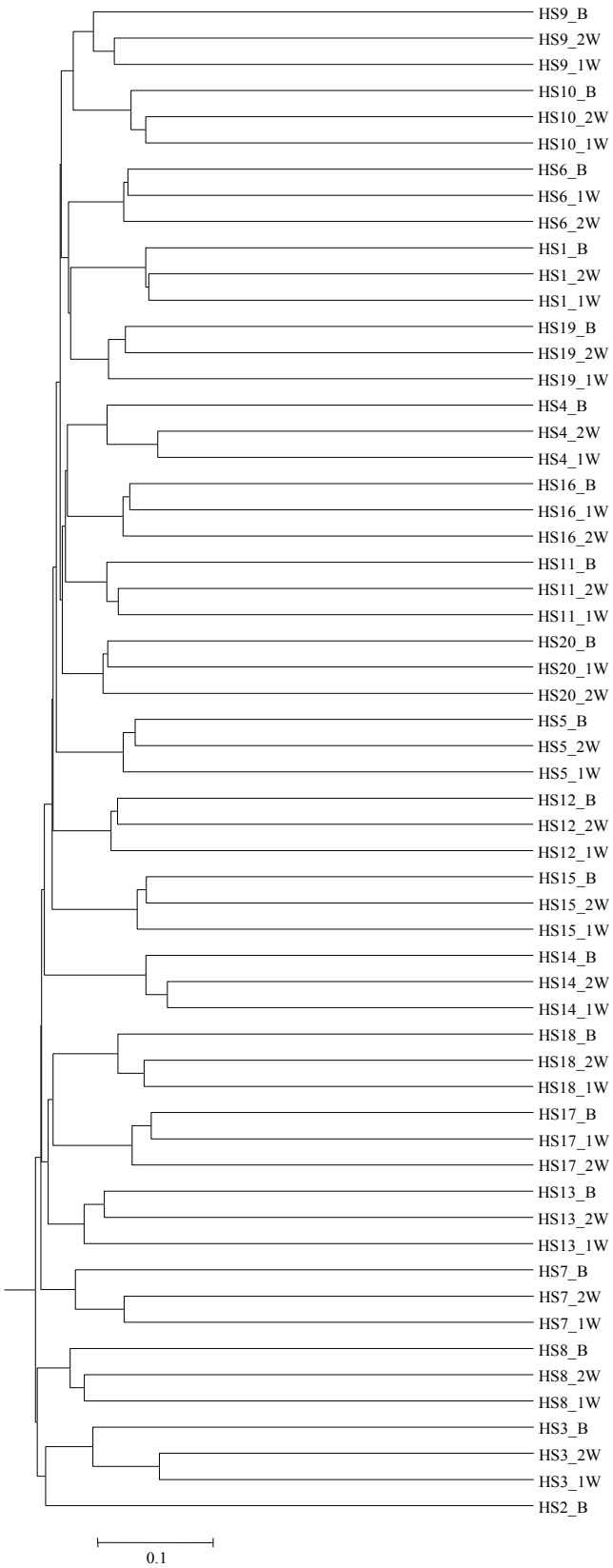

Figure 5: Clustering of plaque communities in experimental gingivitis. Dendrogram of plaque samples from all time points of experimental gingivitis; comparison based on their community membership using the Jaccard index. HS=healthy subject, B=baseline, 1W=one week, 2W=two weeks. Numbers indicate subject number.

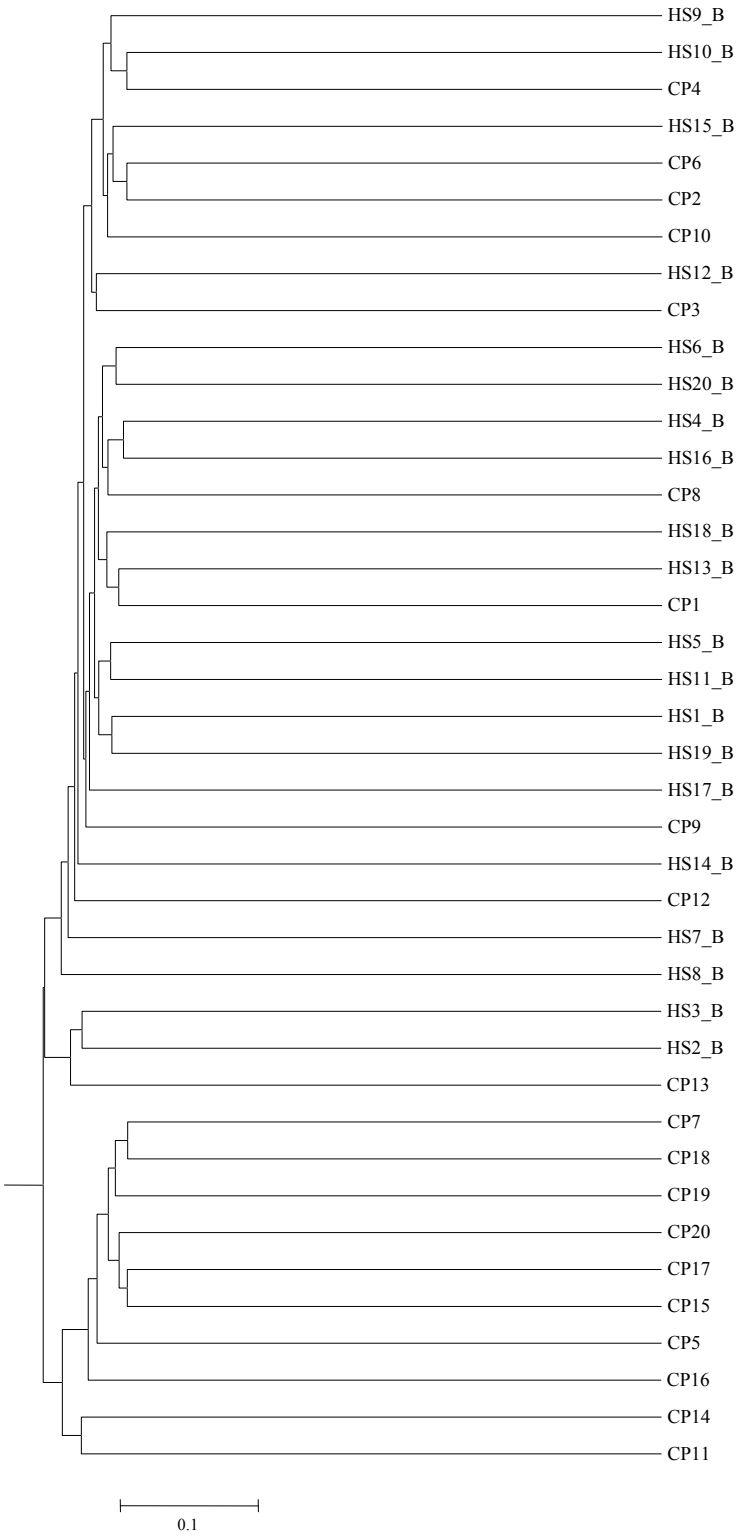

Figure 6: Clustering of plaque communities in health and chronic periodontitis. Dendrogram of plaque samples from the baseline time point of the experimental gingivitis cohort and superficial plaque samples from patients with periodontitis; comparison based on their community membership using the Jaccard index. HS=healthy subject, B=baseline, CP=chronic periodontitis patients. Numbers indicate subject or patient number.

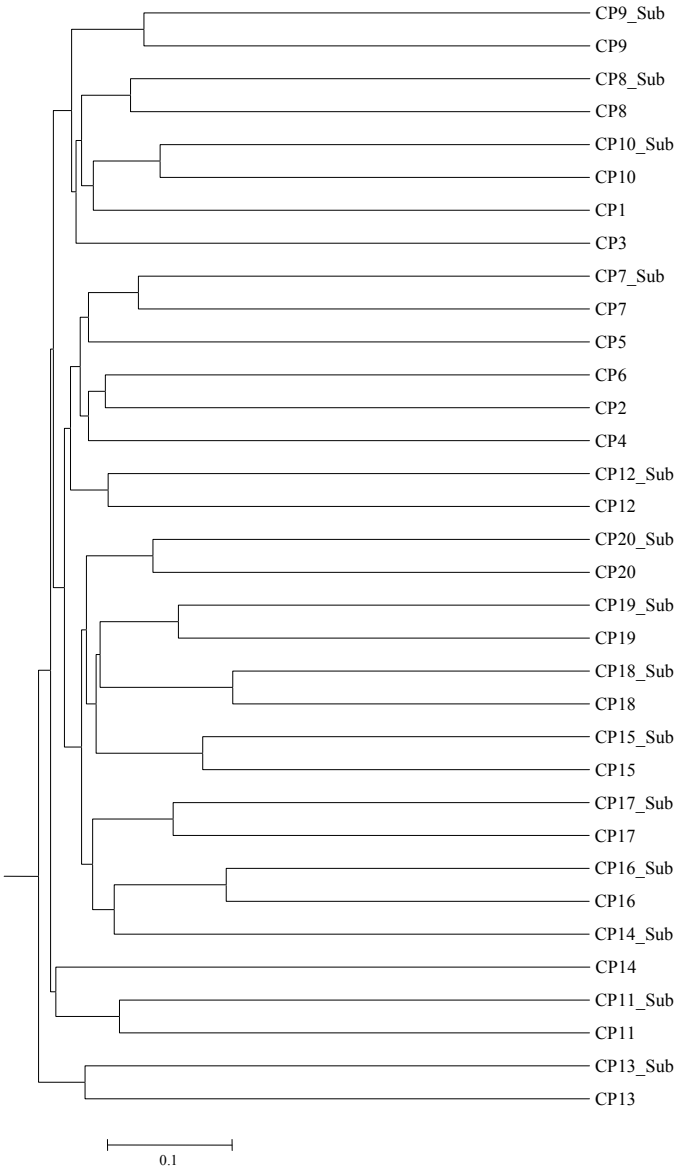

Figure 7: Clustering of superficial and subgingival plaque communities in chronic periodontitis. Dendrogram of superficial and subgingival plaque samples from patients with chronic periodontitis; comparison based on their community membership using the Jaccard index. CP=chronic periodontitis patients, Sub=subgingival plaque. Numbers indicate patient number.

Comparison of the bacterial community structure of plaque samples was performed using the thetaYC and weighted UniFrac metrics from which distance matrices were constructed and visualised using three-dimensional PCoA (Figures 8 and 9). For the experimental gingivitis cohort, both plots showed spatial separation of one- and two-week communities from baseline communities (Figure 8). In addition, separate PCoA analyses comparing superficial periodontitis communities with healthy baseline communities (Figure 9) and two-week gingivitis (not shown) communities showed separation in each case. AMOVA tests found an overall significant difference between the three time points of experimental gingivitis for both the thetaYC and weighted UniFrac distances ($P<0.0016$ and $P<0.001$, respectively). Pairwise AMOVA comparisons showed that there were significant differences between baseline and one-week communities ($P<0.017$ and $P<0.001$ for thetaYC and weighted UniFrac, respectively) and between baseline and two-week communities ($P<0.0006$ and $P<0.001$ for thetaYC and weighted UniFrac, respectively). However, there was no significant difference between one- and two-week communities ($P=0.526$ and $P=0.098$ for thetaYC and weighted UniFrac respectively). AMOVA tests also confirmed that there were significant differences between the baseline communities of healthy subjects and the superficial communities of the periodontitis patients ($P=0.0095$ and $P<0.001$ for thetaYC and weighted UniFrac, respectively). Similarly, there was a significant difference between the two-week communities and the superficial periodontitis samples ($P<0.0005$ for both thetaYC and weighted UniFrac). There was no significant difference in the thetaYC or weighted UniFrac distances between the superficial and subgingival periodontitis samples from periodontitis patients by AMOVA testing.

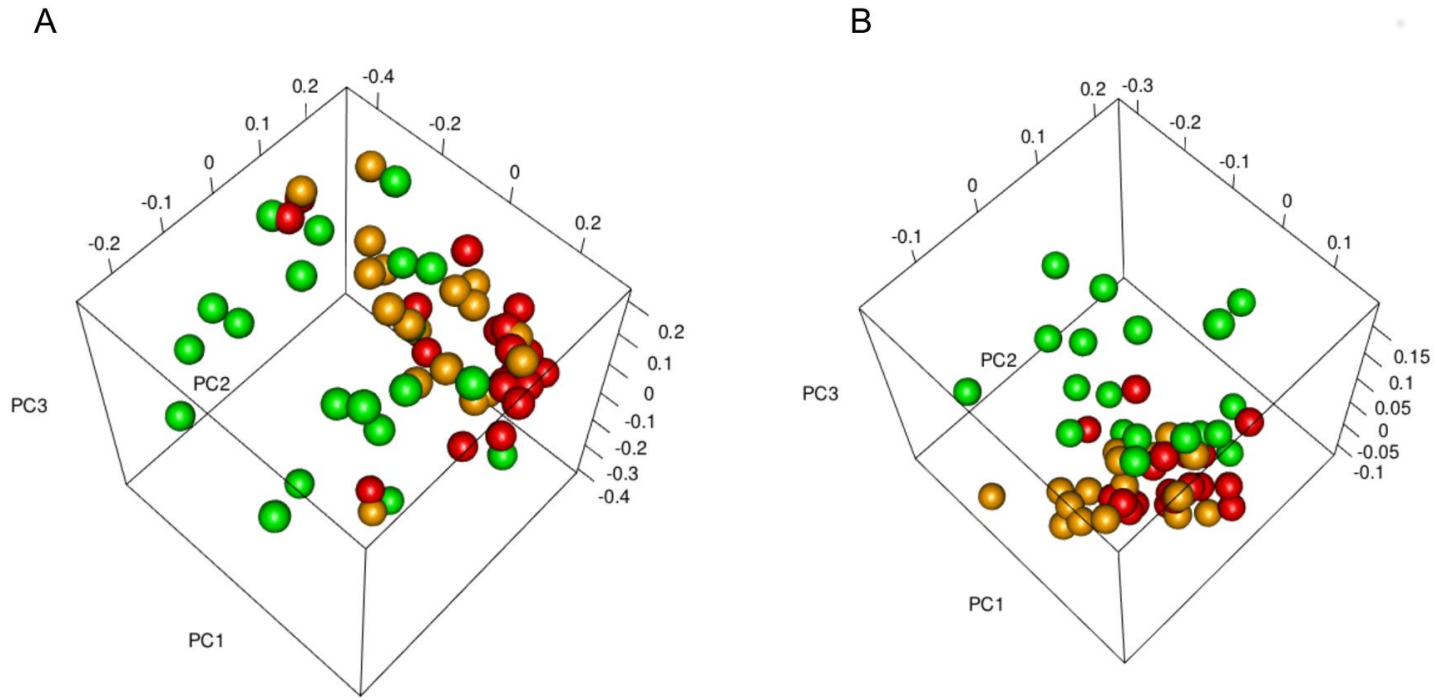

Figure 8: Shifts in the bacterial community structure of plaque during the induction of experimental gingivitis. PCoA plots comparing community structure of plaque samples from different time points of experimental gingivitis. Baseline samples are coloured green, one-week samples are orange and two-week samples are red. (A) PCoA based on the thetaYC calculator. PC1=12.11% of variance explained, PC2=8.89%, PC3=5.55% (B) PCoA based on the weighted UniFrac calculator. PC1=19.78% of variance explained, PC2=10.65%, PC3=5.65%.

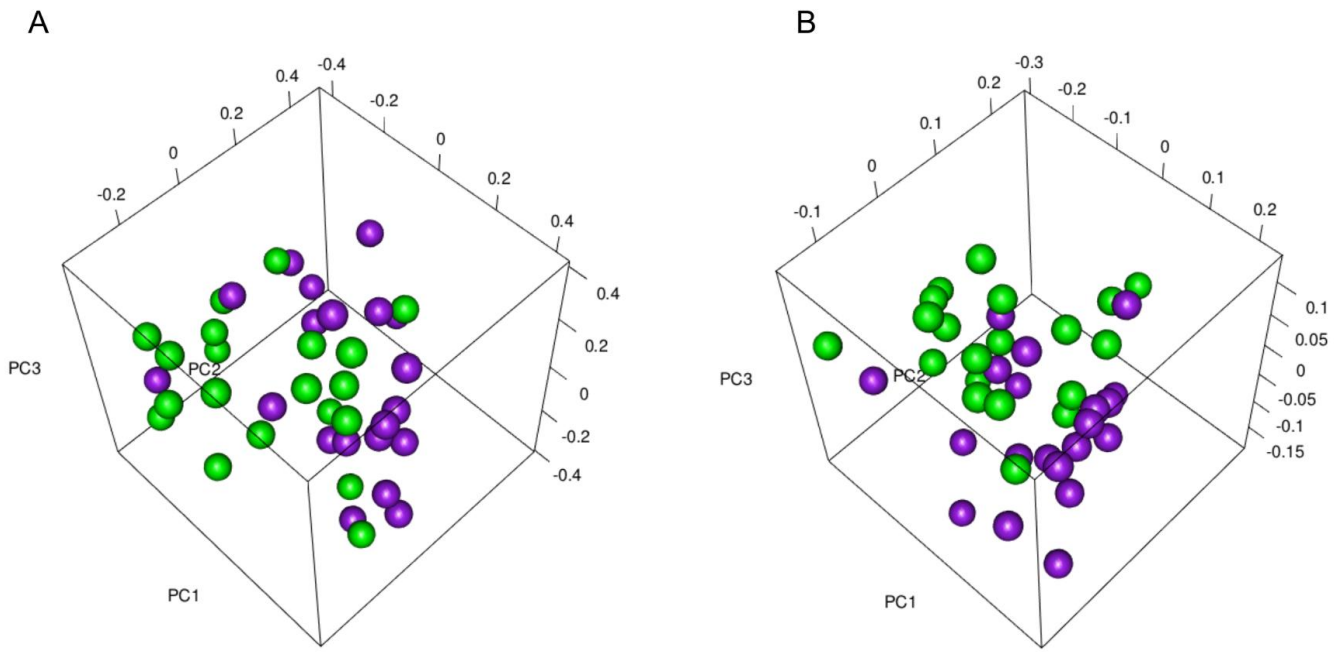

Figure 9: Bacterial community structure of plaque in health and chronic periodontitis. PCoA plots comparing community structure of baseline plaque samples from the healthy cohort to superficial plaque samples of chronic periodontitis patients. Plaque communities from healthy individuals are coloured green and those from periodontitis are coloured purple. (A) PCoA based on the thetaYC calculator, PC1=16.38% of variance explained, PC2=10.09%, PC3=8.02% (B) PCoA based on the weighted UniFrac calculator, PC1=22.12% of variance explained, PC2=8.89%, PC3=6.85%.

2.3.4 *OTU-level composition and shifts of bacterial communities*

The 10 OTUs detected with the highest relative abundance across all 92 samples were assigned to the taxa *Streptococcus sanguinis*, *Rothia dentocariosa*, *Veillonella parvula*, *Fusobacterium nucleatum* subsp. *polymorphum*, *Streptococcus mitis*/HOT064/HOT423/HOTA95/HOTE14, *Streptococcus cristatus*/HOT071, *Fusobacterium nucleatum* subsp. *vincentii*, *Lautropia mirabilis*, *Porphyromonas gingivalis* and *Leptotrichia buccalis*. Statistical analysis using linear models implemented in MaAsLin was initially performed to determine correlations of OTUs with sampling times during experimental gingivitis. The OTUs that were positively or negatively correlated with one- and/or two-week time points of experimental gingivitis are shown in Table 6. Box plots showing changes in relative abundance over time for the most significant OTUs are shown in Figures 10-15. A second analysis in MaAsLin included bleeding on probing (BoP) scores and the baseline and two-week time points. OTUs were statistically associated with the clinical condition indicated by BoP scores, rather than the time point at which the samples were collected. The OTUs identified as *Lautropia* sp. HOTA94, *Lachnospiraceae* sp. HOT100, *Prevotella oulorum* and *Fusobacterium nucleatum* subsp. *polymorphum* were most significantly positively correlated with BoP (P and Q values <0.05) whilst an OTU identified as *Rothia dentocariosa* was most significantly negatively correlated with BoP. A full list of OTUs that were correlated with BoP scores is shown in Table 7. Linear discriminant analysis using LEfSe was used to detect OTUs that had significantly different relative abundances between chronic periodontitis (superficial plaque) and health (baseline plaque). A total of 41 OTUs were found to be significantly differentially abundant between these groups (Figure 16). An OTU

identified as *Porphyromonas gingivalis* was most strongly associated with chronic periodontitis.

Table 6: OTUs associated with time points of experimental gingivitis. OTUs were associated with time points of experimental gingivitis using Multivariate Association with Linear Models (MaAsLin). OTUs are ranked according to their *P* value. OTUs listed have *P* values <0.05.

OTU / Taxon	**Time point**	**Coefficient**	***P* value**	***Q* value**
OTU 0037 *Lautropia* sp. HOTA94	2 weeks	0.074	<0.0002	<0.02
OTU 0118 *Campylobacter showae*	2 weeks	0.051	<0.001	<0.05
OTU 0447 *Prevotella oulorum*	2 weeks	0.032	<0.002	<0.04
OTU 0118 *Campylobacter showae*	1 week	0.049	<0.002	<0.03
OTU 0002 *Rothia dentocariosa*	2 weeks	-0.073	<0.002	<0.04
OTU 0582 *Porphyromonas catoniae*	2 weeks	0.041	<0.003	<0.04
OTU 0020 *Fusobacterium nucleatum* subsp.*polymorphum*	2 weeks	0.061	<0.003	<0.04
OTU 0045 *Actinobaculum* sp. HOT183	1 week	-0.022	<0.006	<0.06
OTU 0199 *Lachnospiraceae* sp. [G-2] HOT100	2 weeks	0.035	<0.009	<0.09
OTU 0008 *Streptococcus mitis*/ HOT064/HOT423/HOTA95/HOTE14	2 weeks	-0.073	<0.01	<0.09
OTU 0113 *Propionibacterium* sp. HOT194	1 week	-0.033	<0.01	<0.08
OTU 0189 *Tannerella* sp. HOT286	2 weeks	0.047	<0.01	<0.08
OTU 0199 *Lachnospiraceae* sp. [G-2] HOT100	1 week	0.034	<0.02	<0.08
OTU 0037 *Lautropia* sp. HOTA94	1 week	0.048	<0.02	<0.09
OTU 0021 *Fusobacterium nucleatum* subsp. *polymorphum*	1 week	0.033	<0.02	<0.09
OTU 0045 *Actinobaculum* sp. HOT183	2 weeks	-0.019	<0.02	<0.08
OTU 0195 *Leptotrichia* sp. HOT212	2 weeks	0.027	<0.02	<0.08
OTU 0002 *Rothia dentocariosa*	1 week	-0.055	<0.03	<0.15
OTU 0027 *Corynebacterium durum*	1 week	-0.032	<0.03	<0.1
OTU 0182 *Leptotrichia* sp. HOT417	2 weeks	0.039	<0.03	<0.15
OTU 0629 *Selenomonas dianae*	2 weeks	0.034	<0.03	<0.1

OTU / Taxon	**Time point**	**Coefficient**	***P* value**	***Q* value**
OTU 0128 *Capnocytophaga sputigena*	1 week	0.045	<0.03	<0.1
OTU 0141 *Actinomyces naeslundii*	1 week	-0.036	<0.03	<0.15
OTU 0020 *Fusobacterium nucleatum* subsp.*polymorphum*	1 week	0.045	<0.04	<0.15
OTU 0066 *Capnocytophaga leadbetteri*	1 week	0.054	<0.04	<0.15
OTU 0364 *Leptotrichia hongkongensis*	2 weeks	-0.047	<0.04	<0.15
OTU 0582 *Porphyromonas catoniae*	1 week	0.029	<0.04	<0.15
OTU 0084 *Gemella morbillorum*	2 weeks	0.048	<0.05	<0.15
OTU 0113 *Propionibacterium* sp. HOT194	2 weeks	-0.026	<0.05	<0.15
OTU 0021 *Fusobacterium nucleatum* subsp. *polymorphum*	2 weeks	0.027	<0.05	<0.15
OTU 0189 *Tannerella* sp. HOT286	1 week	0.037	<0.05	<0.15

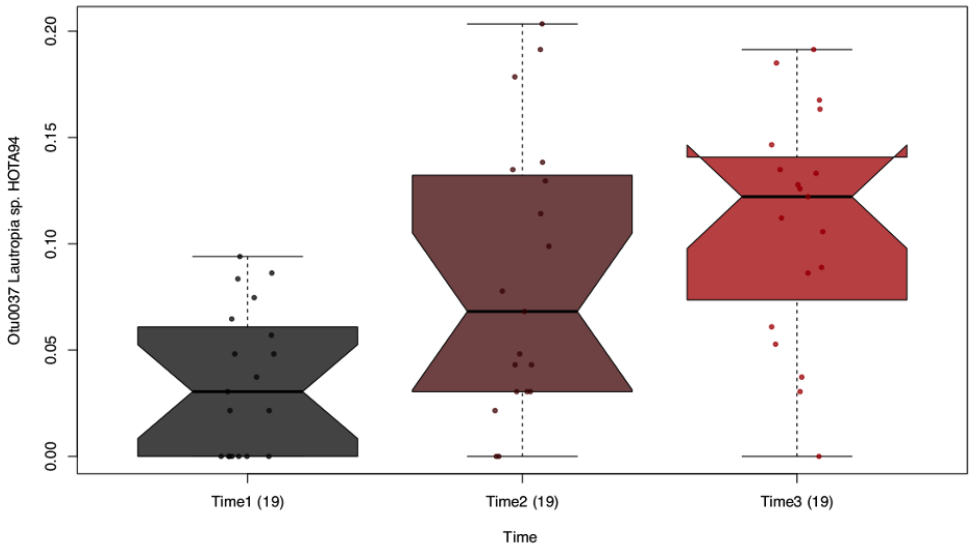

Figure 10: Notched box plot showing change in relative abundance of OTU 37 *Lautropia sp.* HOTA94 in dental plaque during the induction of experimental gingivitis. Time 1 = baseline, Time 2 = 1 week, Time 3 = 2 weeks. Values on y-axis are percentage of total sequences within a sample after sub-sampling.

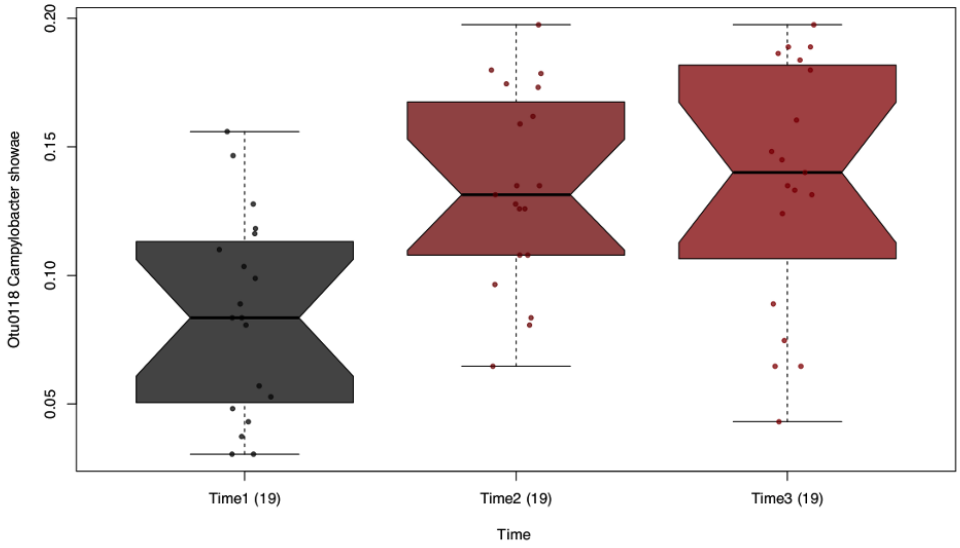

Figure 11: Notched box plot showing change in relative abundance of OTU 118 *Campylobacter showae* in dental plaque during the induction of experimental gingivitis. Time 1 = baseline, Time 2 = 1 week, Time 3 = 2 weeks. Values on y-axis are percentage of total sequences within a sample after sub-sampling.

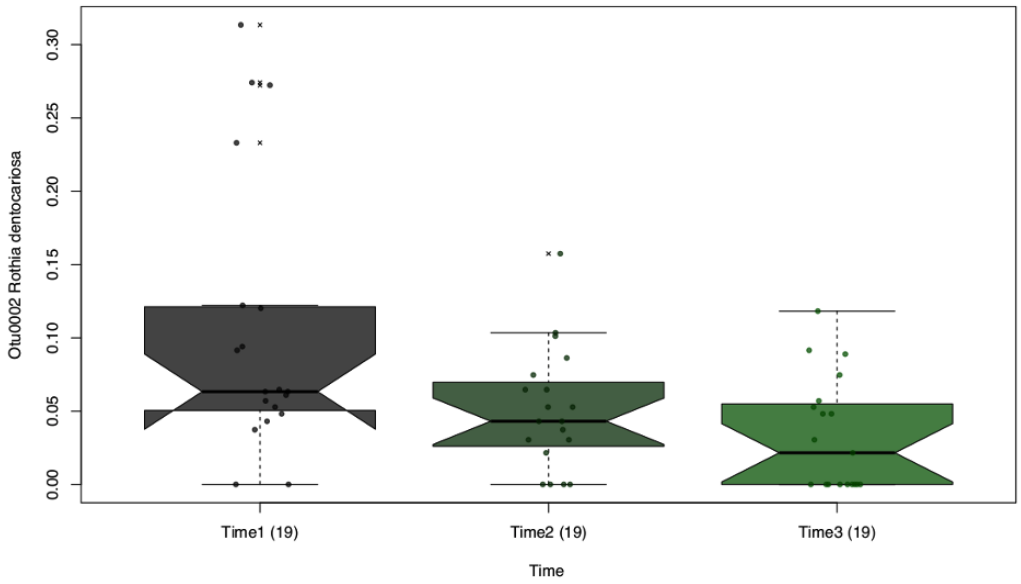

Figure 12: Notched box plot showing change in relative abundance of OTU 2 *Rothia dentocariosa* in dental plaque during the induction of experimental gingivitis. Time 1 = baseline, Time 2 = 1 week, Time 3 = 2 weeks. Values on y-axis are percentage of total sequences within a sample after sub-sampling.

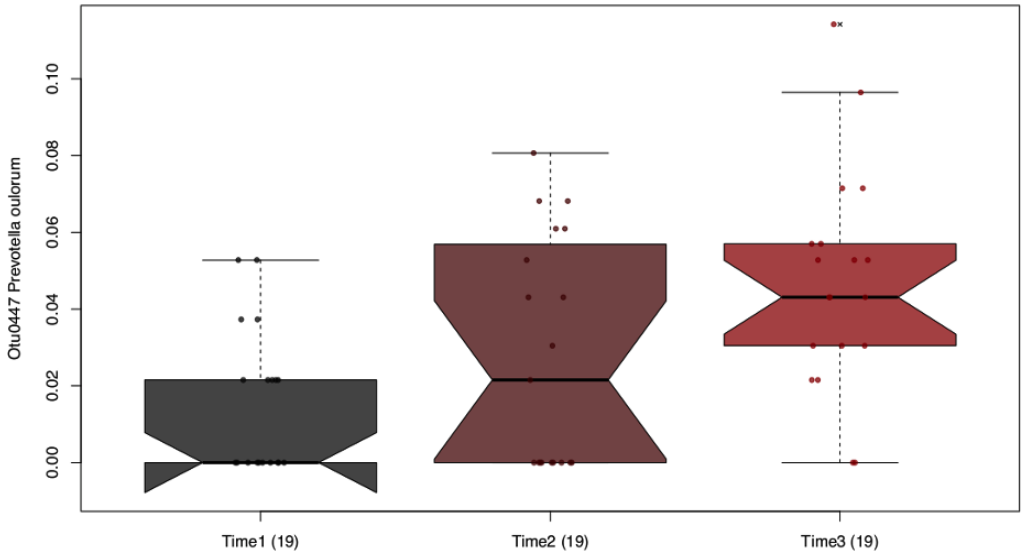

Figure 13: Notched box plot showing change in relative abundance of OTU 47 *Prevotella oulorum* in dental plaque during the induction of experimental gingivitis. Time 1 = baseline, Time 2 = 1 week, Time 3 = 2 weeks. Values on y-axis are percentage of total sequences within a sample after sub-sampling.

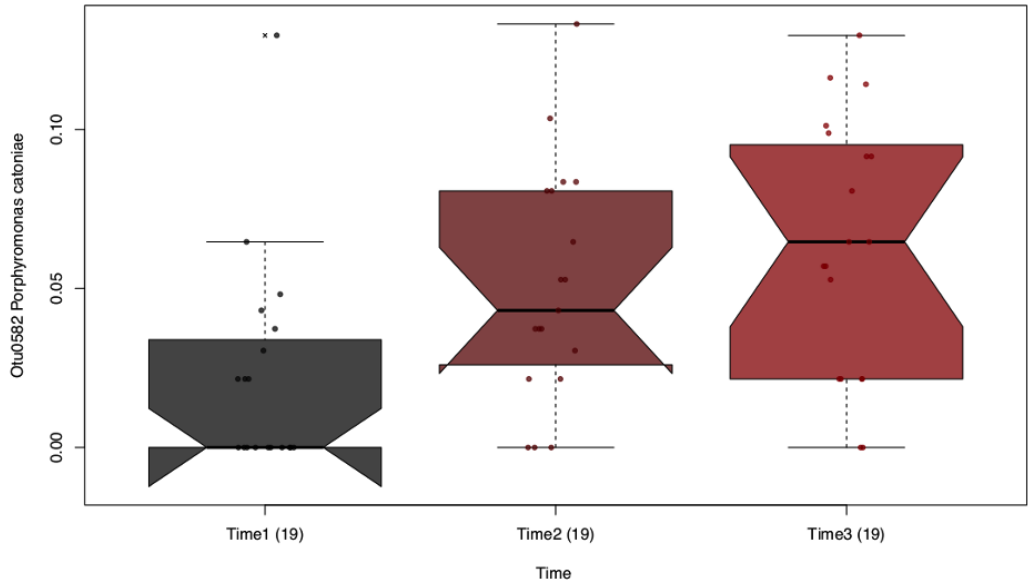

Figure 14: Notched box plot showing change in relative abundance of OTU 582 *Porphyromonas catoniae* in dental plaque during the induction of experimental gingivitis. Time 1 = baseline, Time 2 = 1 week, Time 3 = 2 weeks. Values on y-axis are percentage of total sequences within a sample after sub-sampling.

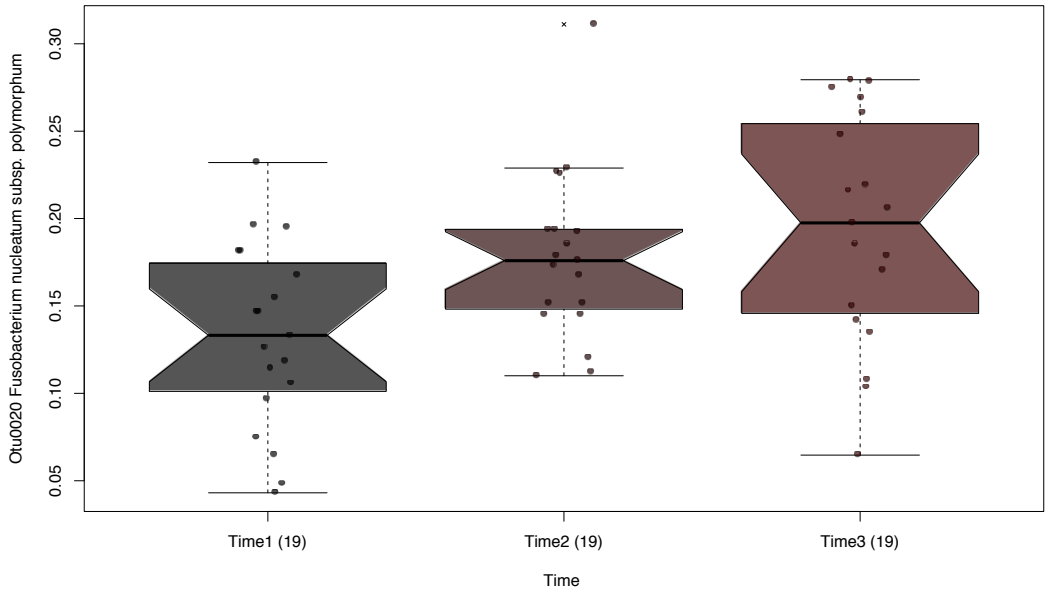

Figure 15: Notched box plot showing change in relative abundance of OTU 20 *Fusobacterium nucleatum* subsp. *polymorphum* in dental plaque during the induction of experimental gingivitis. Time 1 = baseline, Time 2 = 1 week, Time 3 = 2 weeks. Values on y-axis are percentage of total sequences within a sample after sub-sampling.

Table 7: OTUs associated with bleeding on probing (BoP) scores. OTUs were associated with BoP using Multivariate Association with Linear Models (MaAsLin). OTUs are ranked according to their *P* value. OTUs listed have *P* values <0.05.

OTU / Taxon	**Coefficient**	***P* value**	***Q* value**
OTU 0037 *Lautropia* sp. HOTA94	0.0022	<0.00002	<0.004
OTU 0199 *Lachnospiraceae* sp. [G-2] HOT100	0.0011	<0.0004	<0.04
OTU 0447 *Prevotella oulorum*	0.0009	<0.0005	<0.04
OTU 0002 *Rothia dentocariosa*	-0.0025	<0.001	<0.05
OTU 0020 *Fusobacterium nucleatum* subsp. *polymorphum*	0.0020	<0.002	<0.05
OTU 0582 *Porphyromonas catoniae*	0.0012	<0.002	<0.07
OTU 0118 *Campylobacter showae*	0.0014	<0.003	<0.08
OTU 0223 *Solobacterium moorei*	0.0008	<0.005	<0.12
OTU 0182 *Leptotrichia* sp. HOT417	0.0013	<0.006	<0.13
OTU 0014 *Haemophilus parainfluenzae*	-0.0016	<0.02	<0.22
OTU 0027 *Corynebacterium durum*	-0.0010	<0.02	<0.23
OTU 0189 *Tannerella* sp. HOT286	0.0012	<0.02	<0.25
OTU 0045 *Actinobaculum* sp. HOT183	-0.0006	<0.02	<0.28
OTU 0112 *Leptotrichia buccalis*	0.0020	<0.02	<0.28
OTU 0303 *Parvimonas micra*	0.0004	<0.03	<0.27
OTU 0021 *Fusobacterium nucleatum* subsp. *polymorphum*	0.0008	<0.03	<0.26
OTU 0008 *Streptococcus mitis*/HOT064/HOT423/HOTA95/HOTE14	-0.0020	<0.03	<0.25
OTU 0040 *Corynebacterium durum*	-0.0009	<0.03	<0.26
OTU 0364 *Leptotrichia hongkongensis*	-0.0014	<0.04	<0.33
OTU 0066 *Capnocytophaga leadbetteri*	0.0012	<0.04	<0.38
OTU 0033 *Actinomyces* sp. HOT169	-0.0012	<0.05	<0.40

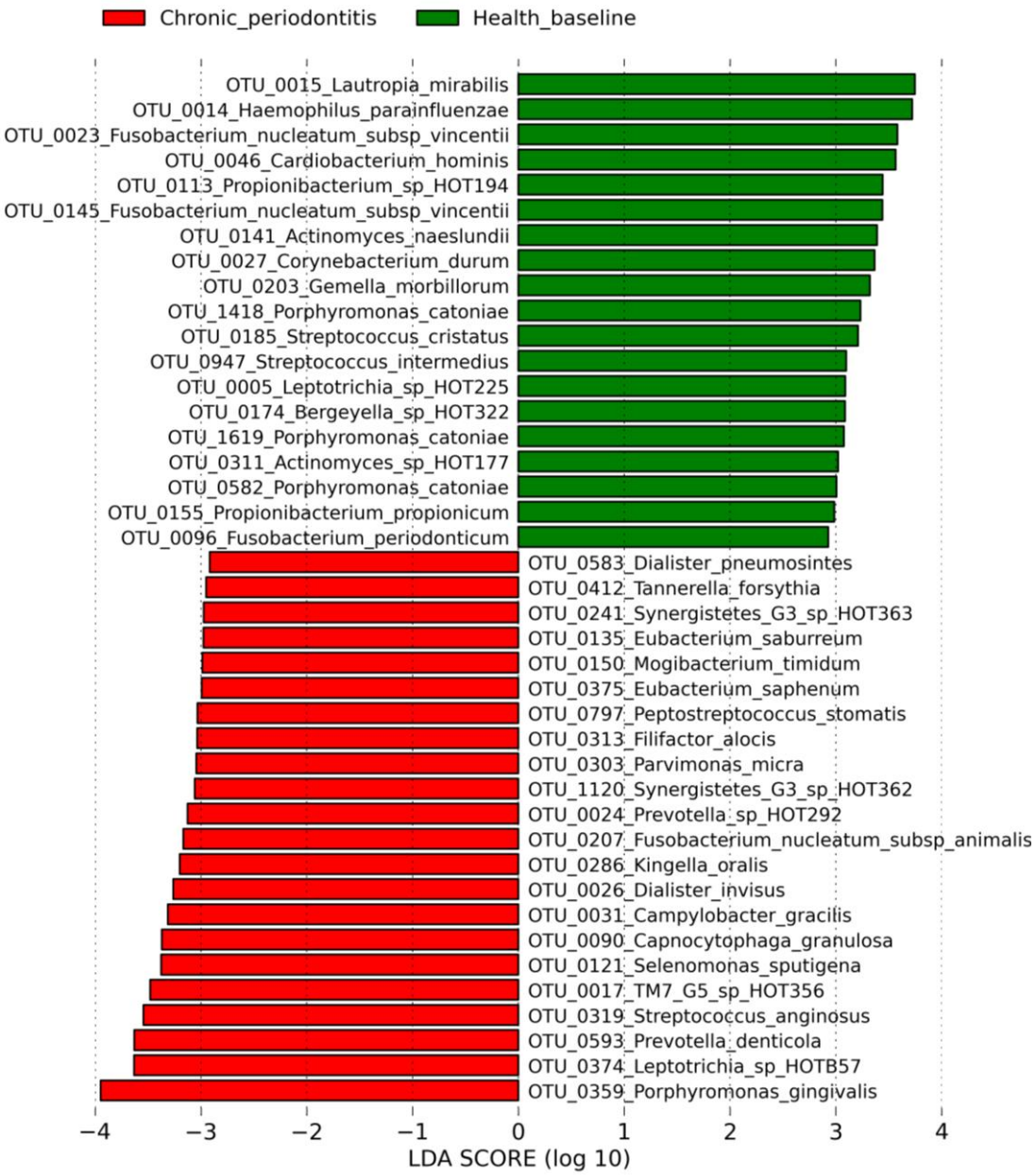

Figure 16: Detection of differentially abundant OTUs in health and chronic periodontitis. Differentially abundant OTUs between baseline plaque communities of the experimental gingivitis cohort and superficial plaque communities in chronic periodontitis patients as identified by LEfSe. OTUs are ranked by their LDA effect size. OTUs associated with healthy subjects are shown in green and OTUs associated with chronic periodontitis are shown in red.

2.3.5 *BLAST identification using the HOMD reference set*

2.3.5.1 BLAST summary

Following BLAST, 331,398 of the 344,267 sequences (96.3%) were mapped to taxa in the HOMD reference set. The 331,398 sequences were assigned to a total of 11 phyla, 126 genera and 567 species-level phylotypes/groups. 3.7% of the 344,267 sequences were not assigned (<98.5%) to any known phylotypes in the HOMD reference set.

2.3.5.2 Phylum level composition and shifts

The predominant phyla detected across all plaque samples in order of mean relative abundance in samples were *Firmicutes* (31.3%), *Fusobacteria* (19.0%), *Bacteroidetes* (18.9%), *Actinobacteria* (14.0%), and *Proteobacteria* (13.9%). Other phyla detected, but which were not present in every sample, included TM7, *Synergistetes*, *Spirochaetes*, SR1, *Chloroflexi* and GN02. There was considerable inter-individual variability in the relative abundance of phyla, both among healthy individuals and patients with chronic periodontitis. However, comparison of the mean relative abundances of the predominant phyla for each cohort (Figure 17) showed that *Proteobacteria* were significantly more abundant in health (two sample *t*-test: $P<0.0056$) whilst *Bacteroidetes* were significantly more abundant in periodontitis ($P<0.0039$). Furthermore, the phyla *Synergistetes* and *Spirochaetes* were detected in 90% and 95% of the periodontitis patients and 40% and 80% of the healthy subjects, respectively. The phylum *Chloroflexi* was not detected in health but found in 15% of the periodontitis patients at low levels. Phylum-level shifts within and across all subjects were observed during experimental gingivitis (Figure 18). Specifically, the relative abundance of *Actinobacteria* was significantly higher at baseline compared to one and two weeks (paired *t*-test: $P<0.001$ for both

comparisons), whilst the *Bacteroidetes* were significantly higher in one- and two-week samples compared to baseline ($P<0.001$ for both comparisons). The shifts in phyla during experimental gingivitis, however, showed considerable variability among individuals. For example, subject four showed a striking drop in their relative abundance of *Actinobacteria*, from 41.6% at baseline to 9.3% after two weeks. In contrast, the relative abundances of *Actinobacteria* in subject five were 15.1% at baseline and 18.5 % after two weeks.

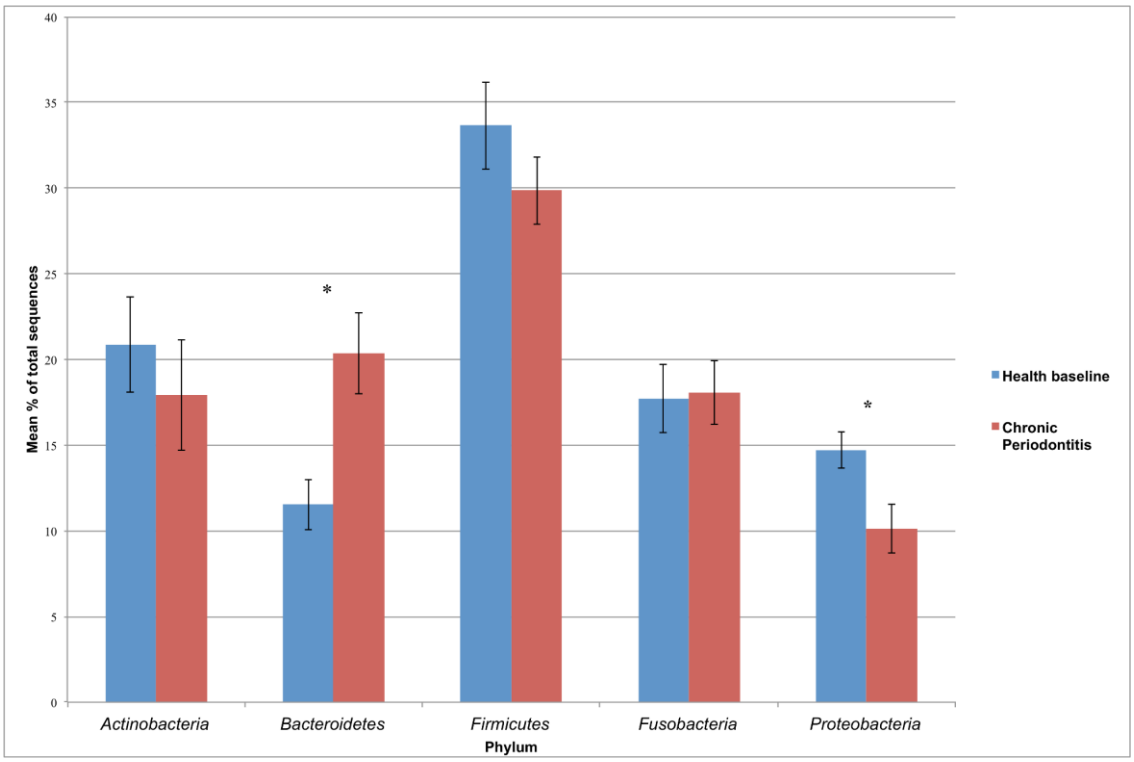

Figure 17: Relative abundances of the predominant phyla in health and chronic periodontitis. Histogram comparing the mean relative abundances of the predominant phyla detected in healthy subjects (baseline) and chronic periodontitis patients (superficial plaque). Statistically significant differences as indicated by two-sample t-tests are highlighted with an * and error bars shown are the standard error of the mean (SEM).

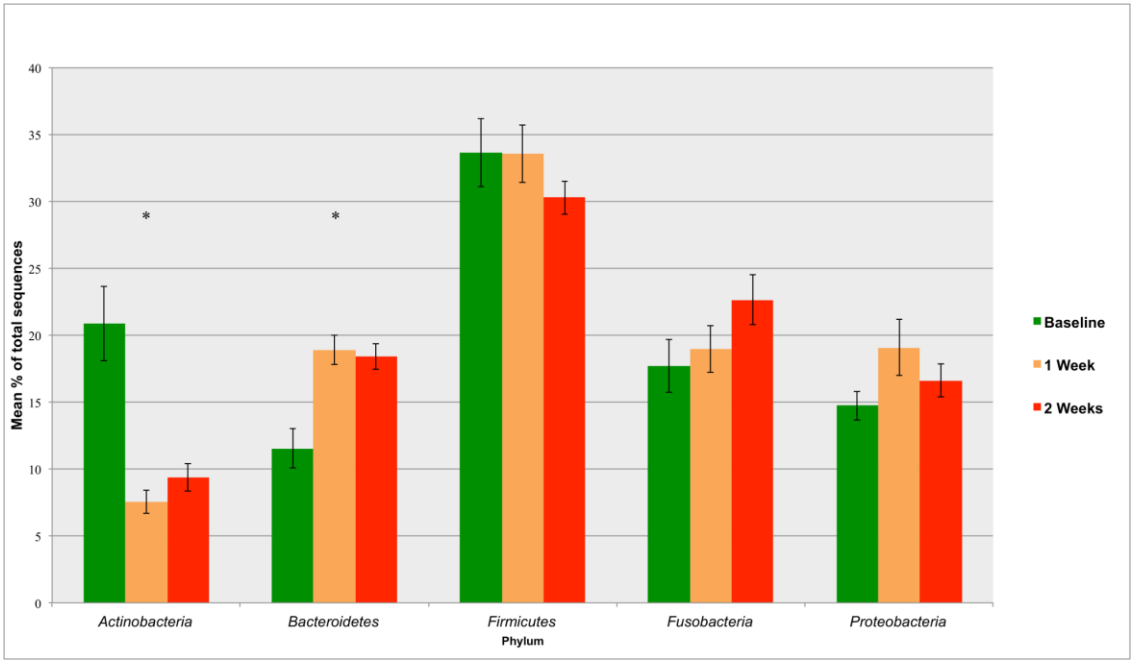

Figure 18: Relative abundances of the predominant phyla during the induction of experimental gingivitis. Histogram chart comparing the mean relative abundances of the predominant phyla at the different time points of experimental gingivitis. Statistically significant differences as indicated by two-sample t-tests are highlighted with an * and error bars shown are the standard error of the mean (SEM).

2.3.6 *Classification of sequences to species-level phylotypes*

Classification of sequences was also performed using a naïve Bayesian classifier implemented in mothur with the HOMD v10.1 reference set. Unlike BLAST analysis, this does not require alignment of the query and reference sequences. Instead, the classifier uses a probability-based algorithm that looks for the presence of specific k-mers (sub-sequence 'words' 8 bases in length) within the sequences (Wang et al., 2007). In addition, a bootstrapping algorithm is incorporated to assess the confidence of the taxonomic assignment. The naïve Bayesian classifier enabled classification of the sequences to species-level in most cases (using a minimum bootstrap confidence threshold of 80%). However, classification to species-level was not possible for the majority of sequences assigned to the genera *Streptococcus* (67.7% of sequences were unclassified) and *Neisseria* (59.8%). A full table detailing

the classifications of the sequences across all of the samples (at seven different taxonomic levels) is provided in a supplementary Microsoft Excel file on the CD-ROM. The most highly represented classified phylotype across all of the plaque samples was *Veillonella parvula*, accounting for 4.5% of the total sequences. Other commonly detected phylotypes that each accounted for >1% of the total number of sequences included: *Actinomyces naeslundii*, *Capnocytophaga leadbetteri*, *Cardiobacterium hominis*, *Campylobacter showae*, *Corynebacterium matruchotii*, *Fusobacterium nucleatum* subsp. *animalis*, *Fusobacterium nucleatum* subsp. *polymorphum*, *Fusobacterium nucleatum* subsp. *vincentii*, *Gemella morbillorum*, *Lautropia mirabilis*, *Leptotrichia hofstadii*, *Porphyromonas gingivalis*, *Prevotella* sp. HOT317, *Prevotella intermedia*, *Rothia dentocariosa*, *Streptococcus* sp. HOT058, and *Streptococcus mitis* biovar.2. BLAST analysis in HOMD enabled provision of the possible alternatives for species-level identifications, where identification using the naïve Bayesian classifier was unsuccessful. Using this method of classification the organism *Streptococcus sanguinis* was the most represented phylotype across the dataset accounting for 5.9% of the total sequences. Other common *Streptococcus* spp. (>1% of total sequences) included the *Streptococcus mitis* group, *Streptococcus cristatus*, and *Streptococcus* sp. HOT058.

2.3.7 *Culture-based community analysis*

Baseline and two-week plaque samples were cultured for 11 of the healthy subjects. For one subject, only the baseline sample was obtained (subject two). 1935 of 1956 isolates were assigned to taxa in the HOMD extended reference set V1.1 using BLAST (≥98.5% sequence identity). These sequences represented five phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria*) and 43 genera. Tables showing the species-level identities (or possible alternatives where

species-level identification was not possible) for all of the isolates obtained from each sample are provided in a supplementary Microsoft Excel file on the CD-ROM. The phylotypes / groups detected with the highest relative abundance using both incubations (anaerobic and air + 5% $C0_2$) were *Streptococcus sanguinis* and the *Actinomyces naeslundii* group. *S. sanguinis* was the most abundant isolate at baseline with a median relative abundance of 18.8% and 13.8% using air + 5% $C0_2$ and anaerobic incubations respectively. After two weeks the *A. naeslundii* group was the most abundant (21.7% and 14.0% using air + 5% $C0_2$ and anaerobic incubations respectively). The observed OTU richness and diversity of baseline and two-week communities by culture is summarised in Table 8.

Table 8: Alpha diversity of plaque samples as analysed by culture.

Time point (incubation)	No. of observed OTUs Median (IQR)	Simpson's inverse diversity index Median (IQR)	Chao 1 total OTU richness estimate Median (IQR)
Baseline (air +5% $C0_2$)	14.5 (11.0-21.0)	10.9 (6.6-17.9)	19.1 (15.4-36.0)
2 Weeks (air +5% $C0_2$)	18.5 (15.3-22.0)	14.6 (9.3-19.5)	30.4 (23.2-43.2)
Baseline (Anaerobic)	22.5 (18.3-24.8)	16.8 (12.7-29.2)	51.3 (31.0-71.2)
2 Weeks (Anaerobic)	24.5 (21.5-28.3)	25.0 (15.8-34.6)	57.9 (38.0-70.4)

A number of taxa isolated in culture were not detected by pyrosequencing among the same samples, including *Actinomyces* sp. HOT172, *Capnocytophaga* sp. HOT380, *Staphylococcus epidermidis* group, *Staphylococcus hominis*, *Staphylococcus warneri*, *Paenibacillus* HOTA06, and *Propionibacterium acnes*/HOT193. No representatives of the phyla GN02, *Spirochaetes*, SR1, *Synergistetes*, or TM7, were detected by culture.

2.3.7.1 16S rRNA gene sequence variation in isolates

Many isolates that were identified as *Actinomyces naeslundii* group species were initially difficult to sequence. Interestingly, close inspection of the sequence electropherograms indicated that the DNA sequence template was mixed, with more than one peak at the same position, despite pure cultures being obtained for identification. One example of this is shown in Figure 19. The same observation was made for other isolates subsequently identified as *Corynebacterium durum*, *Selenomonas sputigena*, and *Prevotella nigrescens*.

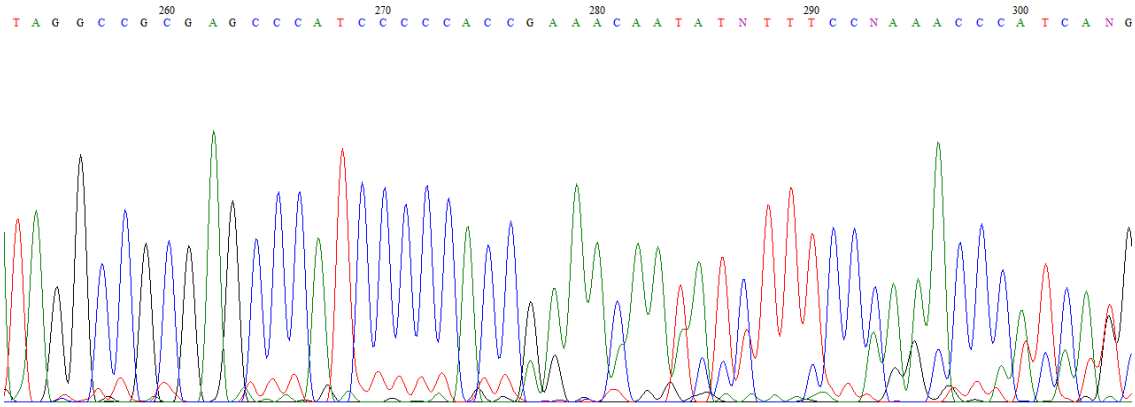

Figure 19: Section of an electropherogram showing a portion of the 16S rRNA gene sequence of isolate HS20_2W_I43.

For those isolates that yielded poor quality mixed sequences, the PCR product was cloned using the TOPO cloning kit and *E. coli* (as described in the methods for novel oral taxa) and multiple clones for each were sequenced using primer 519R (targeting V1-V3). In each case, high quality (unmixed) sequences were obtained and alignment of these sequences confirmed sequence variation in the V1 and V2 regions of different copies of the 16S rRNA gene from the same isolate. One example of this, for the *Actinomyces naeslundii* group isolate HS3_2W_I48 in which variation was particularly high, is shown in the sequence alignment in Figure 19.

Sequencing of 16 clones of this isolate resulted in the detection of three different sequence variants. The first and second variants were each represented by six clones, whilst the third variant was represented by four clones. At a total of 461 bases in length the third variant was the longest and had five-base insertions at bases 192-196 and at bases 200-204 of the alignment. The sequences of variants one and two were the same length (451 bases) as each other but had a total of ten base changes concentrated over two regions of the alignment (see Figure 20). In addition to the cloning approach, isolates yielding mixed sequences were sequenced using primer 1492R as an alternative. There was no variation in the partial 16S rRNA gene sequences (spanning V6-V9) within the same isolates obtained using 1492R, and BLAST identification of the sequences was possible.

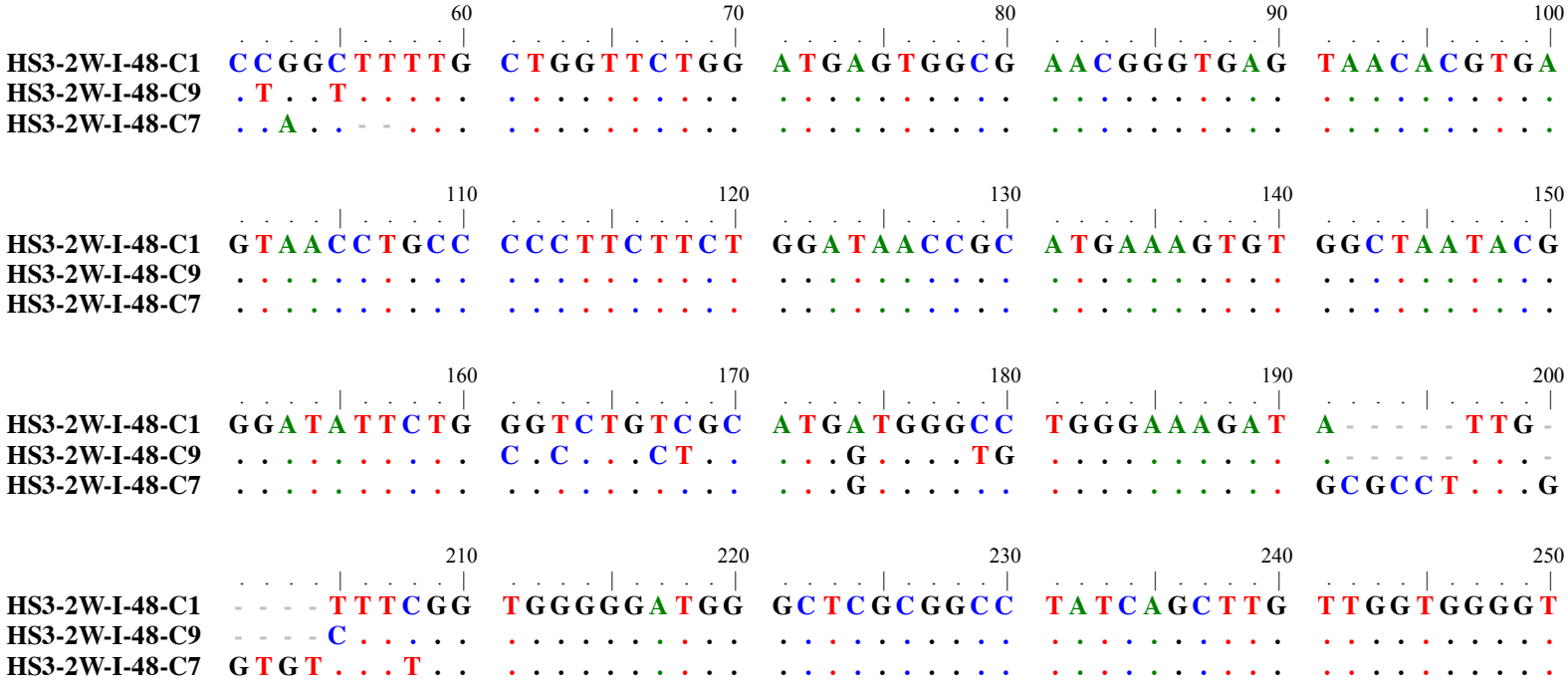

Figure 20: Section of an alignment of three 16S rRNA gene sequences cloned from isolate HS3_2W_I48 (*Actinomyces naeslundii* group) showing sequence copy variation in the V1 and V2 regions. Clones representing three different variants are shown in the alignment. C1 is representative of variant 1, C9 is representative of variant 2 and C7 is representative of variant 3.

2.3.8 *Novel oral taxa*

12,869 sequences in the pyrosequencing dataset were not mapped to any taxa in HOMD (<98.5% sequence similarity). These sequences represented lineages within a number of different phyla and many were found in multiple samples in both the experimental gingivitis and chronic periodontitis cohorts. Seven of these phylotypes/groups were investigated further. Virtually full-length sequences were obtained for all of these phylotypes/groups using specific 16S rRNA primers for targeted PCR, as well as for two novel cultured isolates and made available on GenBank and HOMD. A summary of these taxa, their closest known phylogenetic relatives, and Genbank accession numbers are shown in Table 9. Of particular interest was a deep branching lineage within the class *Mollicutes* representing a new order. The prevalence of these taxa in the experimental gingivitis and chronic periodontitis cohorts is shown in Table 10 and phylogenetic trees for each taxon are shown in Figures 21-29.

Table 9: Oral taxa targeted for full-length 16S rRNA gene sequencing.

Closest phylogenetic relative in HOMD (% sequence identity)	Name and accession no. of closest match in Genbank (% sequence identity)	Clone/Isolate	Genbank accession no.	HOMD oral taxon no.
Bacillus sp. HOT A03 (84%)	Uncultured bacterium clone 4_11, HE681226 (99%)	Clone	KC203059	HOT 906
Microlunatus sp. HOT C95 (93.5%)	Uncultured *Propionibacteriaceae* bacterium clone 08_3_G04, GU227180 (99%)	Clone	KC203064	HOT 915
Prevotella sp. HOT 473 (94.3%)	Uncultured *Prevotellaceae* bacterium clone 601F05, AM420222 (99%)	Clone	KC203063	HOT 914
Actinomyces sp. HOT 449 (94.8%)	Uncultured bacterium clone 070050_018, JQ466816 (99%)	Clone	KC203057	HOT 897
Bergeyella sp. HOT 322 (96.3%)	Uncultured bacterium clone rRNA004, AY958777 (99%)	Clone	KC203058	HOT 900
Tannerella sp. HOT 808 (97.9%)	Uncultured *Tannerella* sp. clone 402C09, AM420141 (99%)	Clone	KC203065	HOT 916
Leptotrichia hofstadii (97.9%)	Uncultured *Leptotrichia* sp. clone 303F08, AM420110 (99%)	Clone	KC203062	HOT 909
Aggregatibacter sp. HOT 513 (97.9%)	Uncultured bacterium clone P1D1-738, EF511870 (98%)	Isolate	KC203060	HOT 898
Capnocytophaga sp. HOT 336 (98.3%)	*Capnocytophaga* sp. P2 oral strain P4_P12, AY429469 (98%)	Isolate	KC203061	HOT 903

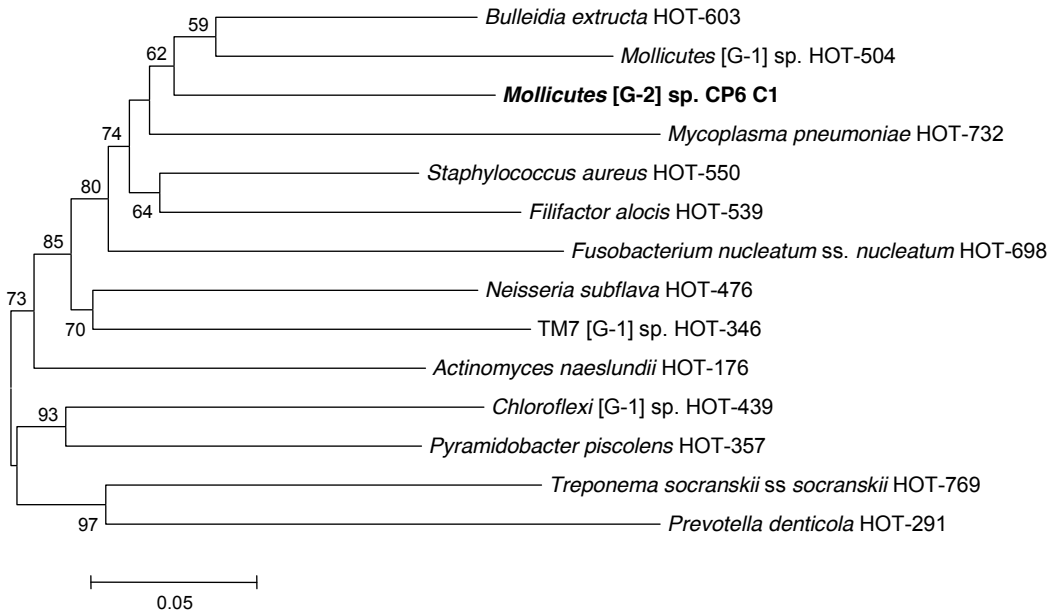

Figure 21: Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Mollicutes*_CP6_C1, members of the *Firmicutes* phylum and other phyla found in the oral cavity. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

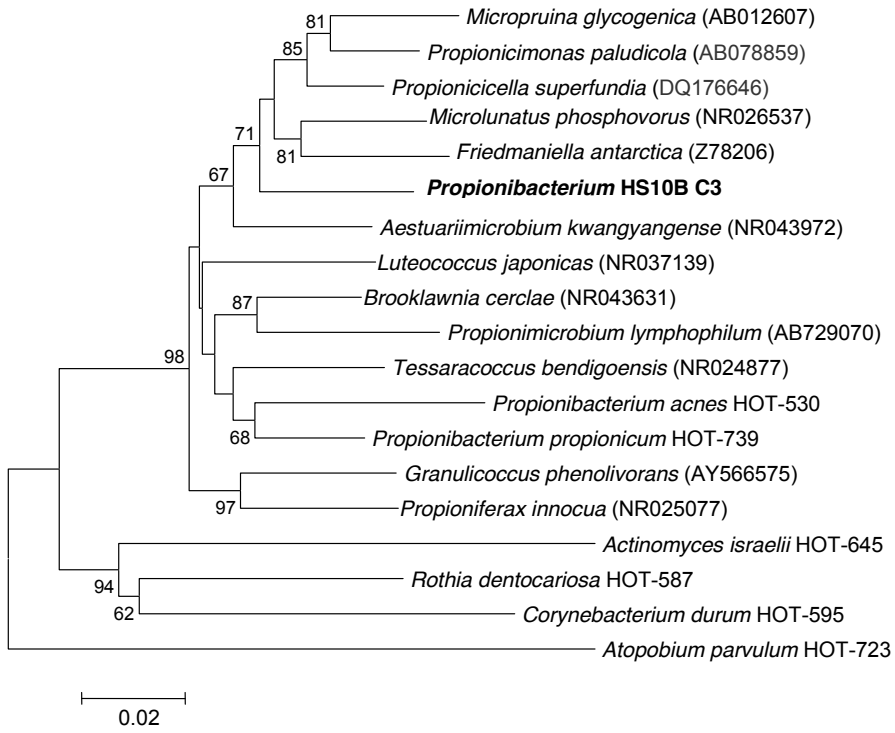

Figure 22: Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Propionibacteriaceae* HS10_B_C3 and members of the phylum *Actinobacteria*. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

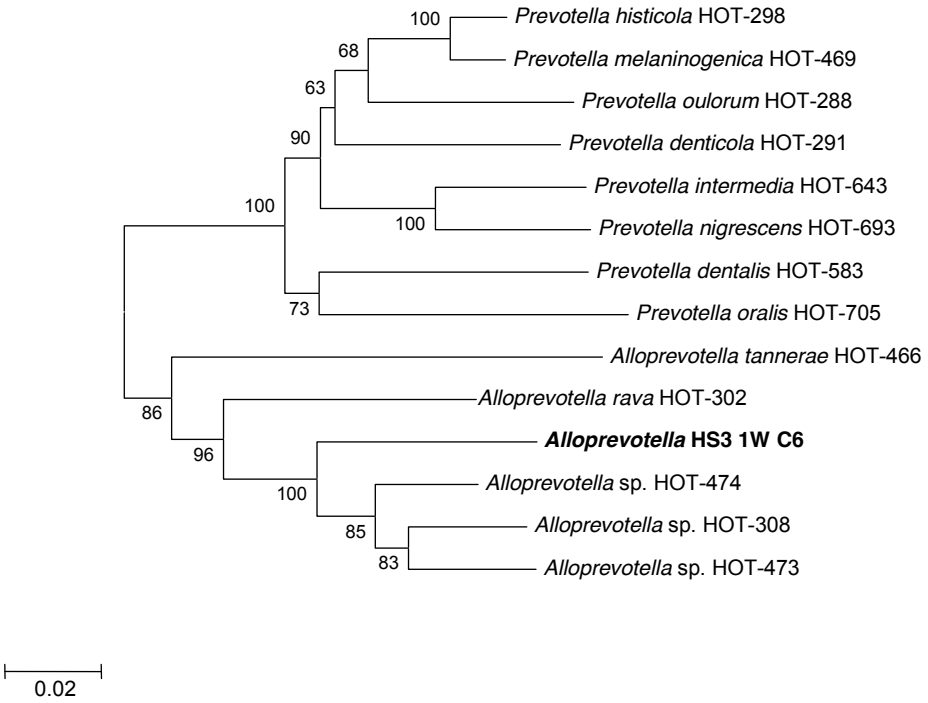

Figure 23: Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between, *Alloprevotella*_HS3_1W_C6 and members of the genera *Alloprevotella* and *Prevotella*. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

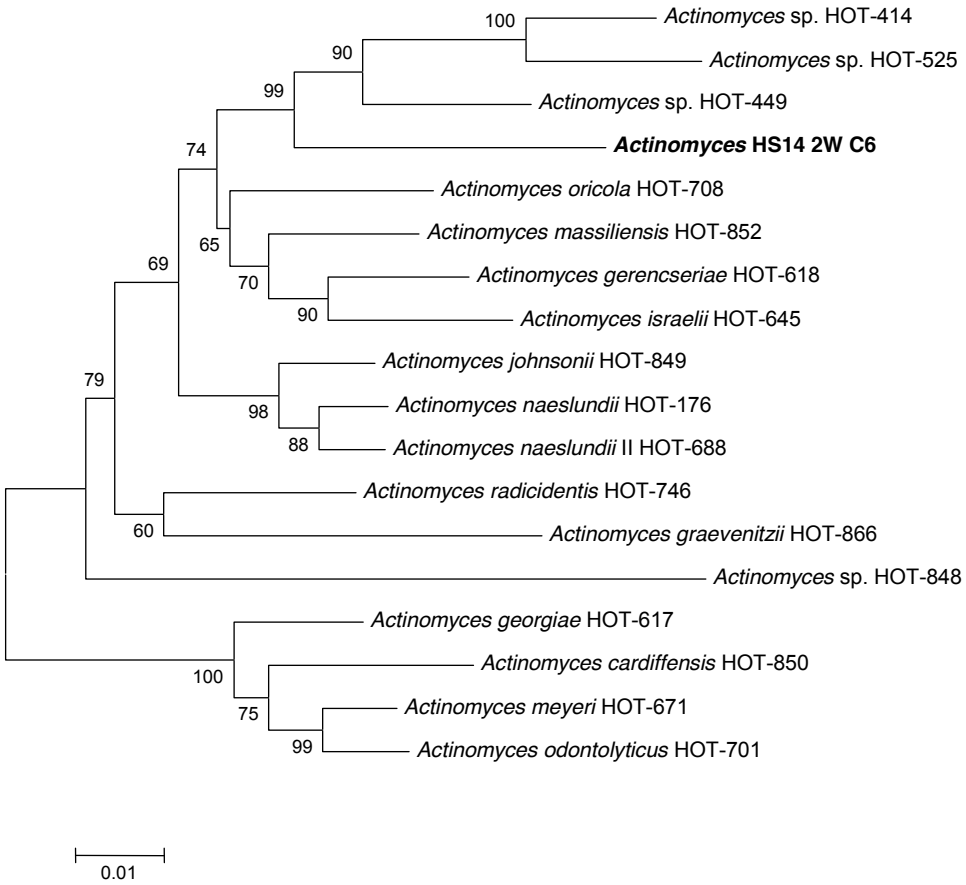

Figure 24: Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between, *Actinomyces*_HS14_2W_C6 and members of the genus *Actinomyces*.The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

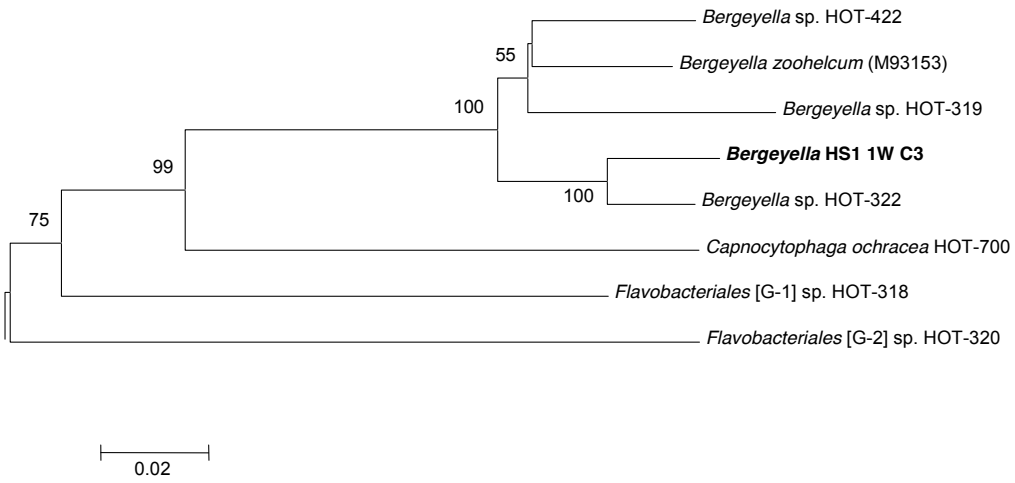

Figure 25: Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Bergeyella*_HS1_1W_C3, members of the genus *Bergeyella* and other members of the class *Flavobacteria* in the phylum *Bacteroidetetes*. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

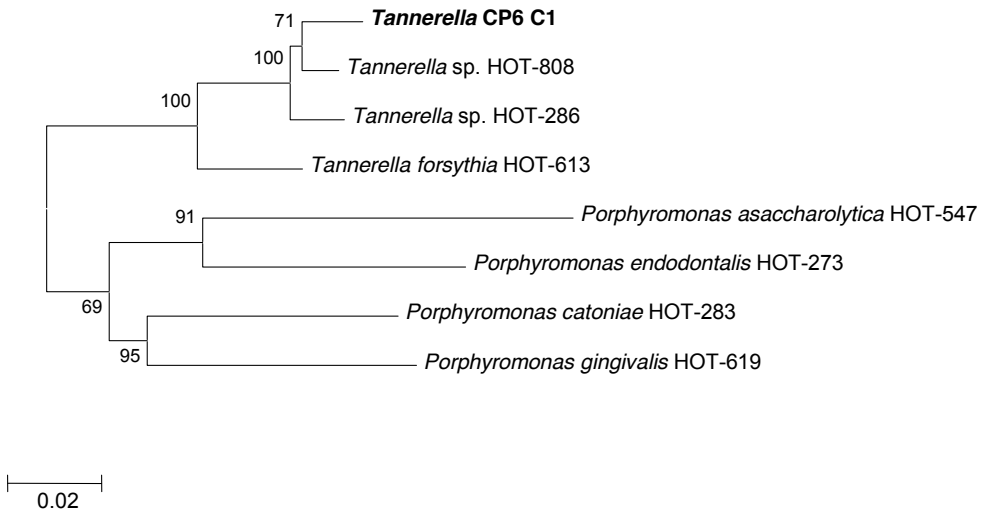

Figure 26: Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Tannerella* CP6_C2 and members of the genera *Tannerella* and *Porphyromonas*. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

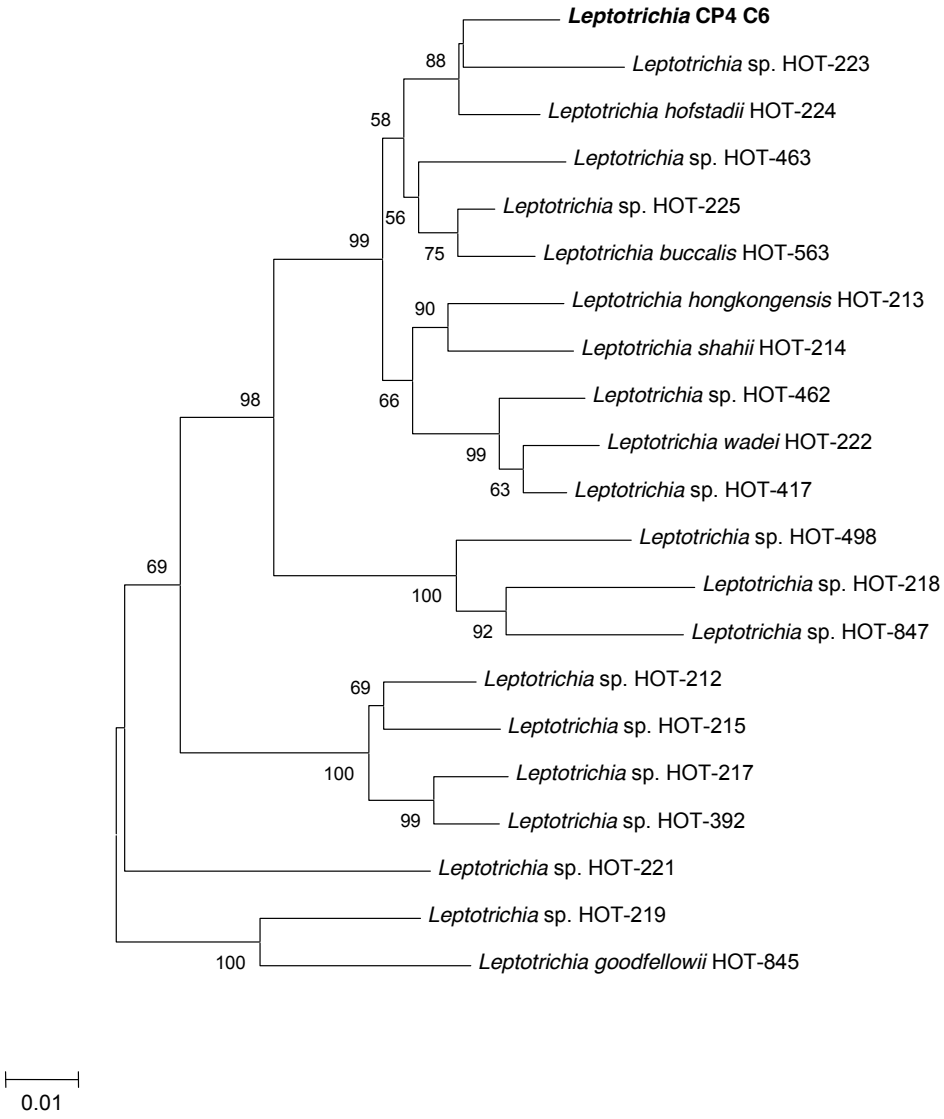

Figure 27: Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Leptotrichia* CP4_C6 and members of the genus *Leptotrichia*. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

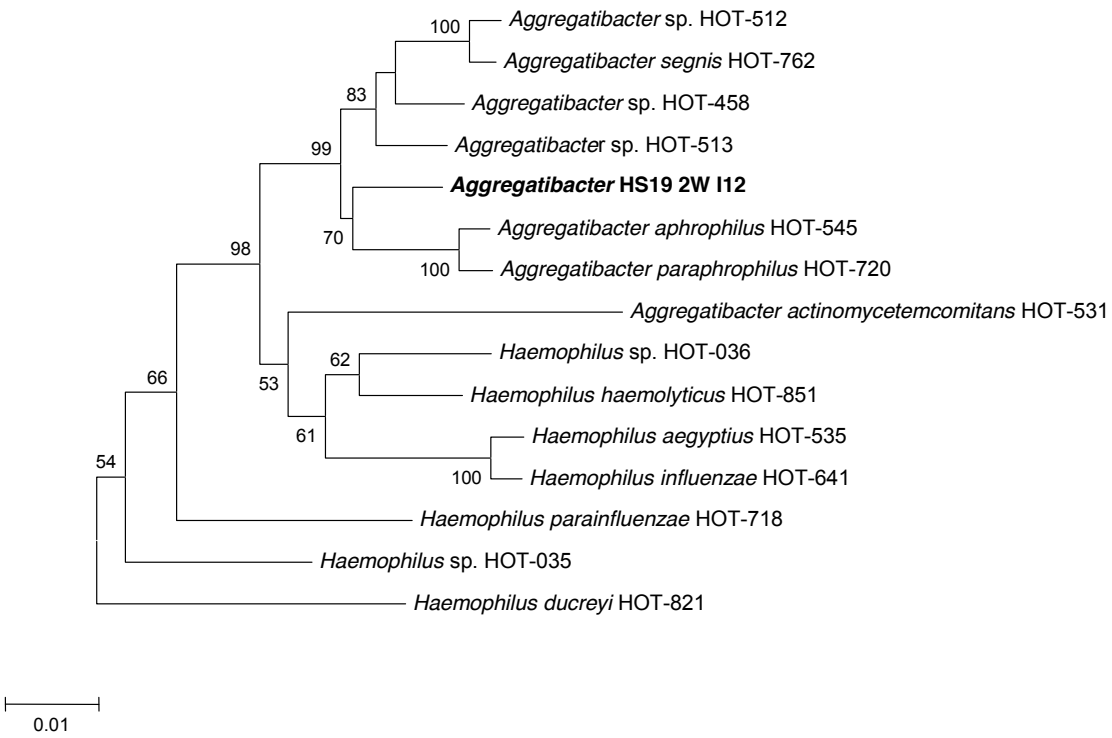

Figure 28: Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Aggregatibacter*_HS19_2W_I12 and members of the genera *Aggregatibacter* and *Haemophilus*. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

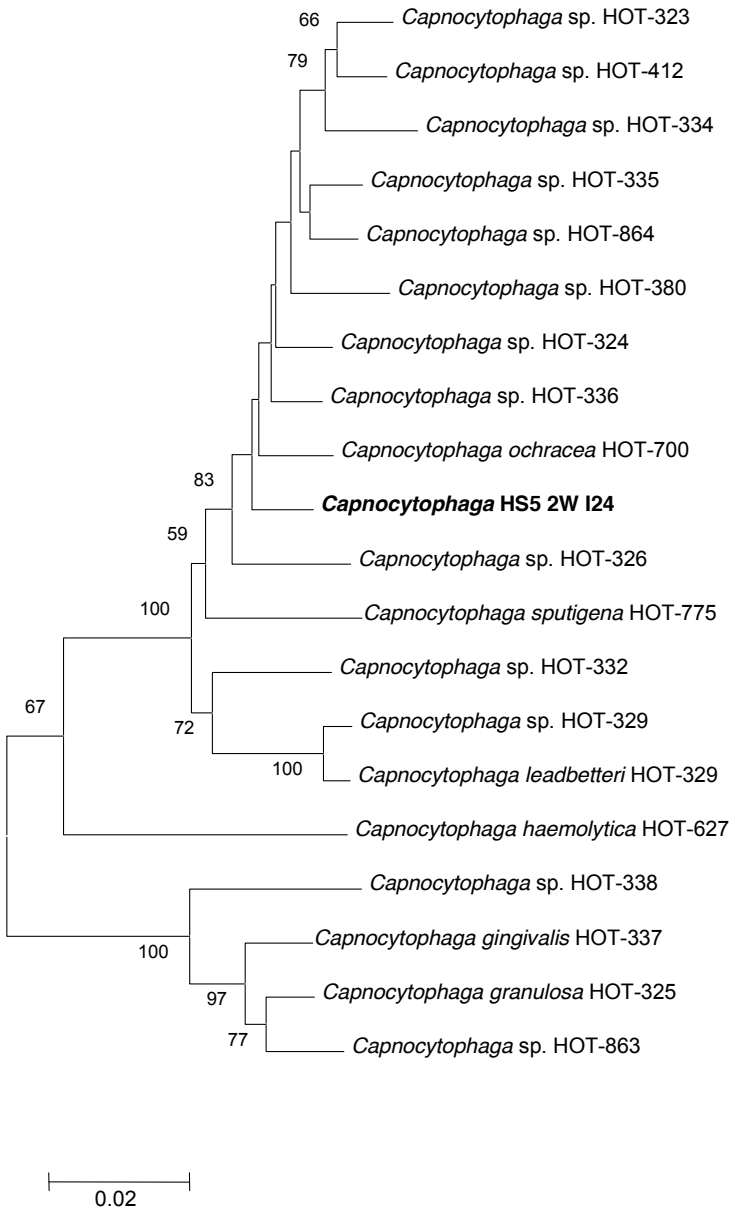

Figure 29: Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Capnocytophaga* HS52WI24 and members of the genus *Capnocytophaga*. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

Table 10: Prevalence of novel taxa in the experimental gingivitis and chronic periodontitis cohorts.

Assigned HOMD taxon name	No. of samples in which phylotype was detected (%)				
	Experimental gingivitis			Chronic periodontitis	
	Baseline (*n*=20)	1 week (*n*=19)	2 weeks (*n*=19)	Superficial plaque (*n*=20)	Subgingival plaque (*n*=14)
Mollicutes sp. HOT906	0 (0)	0 (0)	0 (0)	2 (10)	1 (7)
Propionibacterium sp. HOT915	3 (15)	0 (0)	2 (11)	1 (5)	0 (0)
Alloprevotella sp. HOT914	0 (0)	4 (21)	4 (21)	1 (5)	1 (7)
Actinomyces sp. HOT897	7 (35)	5 (26)	8 (42)	7 (35)	2 (14)
Bergeyella sp. HOT900	2 (10)	4 (21)	2 (11)	2 (10)	2 (14)
Tannerella sp. HOT916	2 (10)	1 (5)	2 (11)	6 (30)	3 (21)
Leptotrichia sp. HOT909	1 (5)	2 (11)	2 (11)	1 (5)	0 (0)
Aggregatibacter sp. HOT898	1 (5)	4 (21)	4 (21)	1 (5)	0 (0)
Capnocytophaga sp. HOT903	1 (5)	0 (0)	1 (5)	0 (0)	0 (0)

2.4 Discussion

This is the first reported use of 454-pyrosequencing to examine the bacterial composition of dental plaque in experimental gingivitis, and one of few longitudinal investigations of the oral microbiome. The results have shown that, in the absence of oral hygiene, the transition from periodontal health to gingivitis is accompanied by a shift in the bacterial community structure of plaque and an increase in bacterial community diversity. The results also demonstrated significant differences in both the membership and structure of analogous health- and chronic periodontitis-associated plaque samples, and confirmed the association of particular species that have previously been associated with chronic periodontitis (Griffen et al., 2012, Abusleme et al., 2013).

A number of previous high-throughput 16S rRNA sequencing studies have characterised oral bacterial communities to the phylum or genus level only (Keijser et al., 2008, Lazarevic et al., 2009, Li et al., 2010). It is important to distinguish taxa at the species-level, as different species within the same phylum and/or genus may be health-associated or pathogenic/disease-associated. For instance, in the oral cavity *Streptococcus mutans* has previously been associated with dental caries (Becker et al., 2002, Tanner et al., 2011), whilst *Streptococcus salivarius* has been associated with oral health (Kazor et al., 2003). The targeting of a highly variable region of the 16S rRNA gene (regions V1-V3) and the use of a curated human oral 16S rRNA gene reference set (HOMD), enabled the identification of OTUs (clustered at a distance of 0.015) and sequences to species-level where possible. Whilst some studies (Huang et al., 2011, Griffen et al., 2012, Abusleme et al., 2013) have also recently reported species-level 16S rRNA gene pyrosequencing analysis of the bacterial communities in periodontal health, gingivitis and chronic periodontitis,

these studies were cross-sectional in nature and did not examine changes in the same individuals during the transition from health to disease. In the present study a highly species-rich bacterial community (201-383 OTUs per sample) was revealed in early health-associated plaque. This richness is considerably higher than indicated by the culture data of this study (Table 8) and in previous studies characterising the oral microbiome in health. Aas et al. (2005) found between 12 and 27 species-level phylotypes on tooth surfaces and between four and 21 in subgingival plaque, while Bik et al. (2010) detected between 65 and 128 species-level OTUs in pooled samples from different oral surfaces. The number of species-level OTUs per plaque sample observed in health in the present study is in a similar range to other recent 16S rRNA pyrosequencing studies. Zaura et al. (2009) found on average 266 species-level phylotypes (97% sequence similarity) per sample and Griffen et al. (2012) detected between 100 and 300 phylotypes (98% sequence similarity) per individual. However, Huang et al. (2011) reported the presence of 379-684 species-level OTUs (97% sequence similarity) in the supragingival plaque of healthy individuals. Comparison of the numbers of observed OTUs, or phylotypes, between this and other studies, though, is complicated by differing methodologies such as, but not limited to, the use of different regions of the 16S rRNA gene and different sequence dissimilarity cut-offs for OTU clustering or BLAST classification. Whilst many studies have used a distance of 0.03 for species-level OTU clustering, this work used a 0.015 cut-off as many common oral bacterial species, particularly the viridans streptococci, are phylogenetically closely related (Do et al., 2009). One problem associated with accurately determining the species richness in oral samples is related to the technical limitations of pyrosequencing. A recent study (Diaz et al., 2012) analysed a mock bacterial community using pyrosequencing and showed that despite de-noising and

stringent quality filtering, additional erroneous OTUs were detected, indicating that pyrosequencing may still over-estimate the number of OTUs present.

The species-level OTU richness detected by pyrosequencing was not significantly higher in subjects after one and two weeks without oral hygiene. This was perhaps surprising given findings to the contrary in a previous culture-based experimental gingivitis study (Moore et al., 1982). These observations are, however, similar to that of Huang et al. (2011) who, using pyrosequencing, did not note any significant difference in richness between plaque samples from healthy individuals and those with gingivitis. The significant increase in community diversity (Simpson's inverse diversity index), after two weeks of experimental gingivitis, in the absence of significantly increased richness, indicated that the increased diversity was mainly a result of increasing evenness. This suggests that as plaque accumulated, species that dominated the early communities in health decreased in relative abundance over time whilst previously minor constituent species increased in relative abundance, resulting in a more even distribution of species after two weeks of plaque accumulation. Significant differences between the bacterial community structures (using both OTU- and phylogenetic-based analyses) of plaque in health (baseline) and gingivitis (one- and two-week plaque samples) were shown by the pyrosequencing data. However, clustering comparisons on the basis of community membership indicated that inter-individual differences were greater. This was supported by the low number of OTUs that were shared between all 20 healthy subjects, suggesting a small core/shared oral microbiota in plaque at this taxonomic level. Previous studies have also found large inter-individual variations in the oral microbiome, although more taxa were shared when considered at the genus or phylum level compared to the species-level (Zaura et al., 2009, Bik et al., 2010). This

large species-level variation between individuals supports the concept of functional redundancy among members of the plaque community, implying that a number of different species may fill the same niche or functional role within the community. Comparison of communities based on membership alone, however, does not take into account the relative abundance of the OTUs present, only their presence or absence in a community.

The PCoA plots revealed a shift in bacterial community structure as gingivitis developed in the subjects following the withdrawal of oral hygiene. It is interesting to note, however, that the PCoA plots also indicated considerable variability in community structure among the healthy subjects. This variability was evident both at the phylum- and species-level. For example, the relative abundance of *Actinobacteria* ranged between 8.4% and 55.9% in baseline samples. At the species-level, OTUs that were dominant in some individuals' baseline samples were detected only at low relative abundances, or not at all, in others. An OTU identified as *Neisseria flavescens* / *subflava* was the dominant OTU at baseline in subject 16 (8.8% of the sequences) but was not detected in 11 of the healthy subjects. It would be useful if future studies examining the microbial composition of plaque in health and periodontal disease include a greater number of subjects, as the relatively low number (20 healthy subjects and 20 chronic periodontitis patients) in the present work was a limitation given the observed inter-individual variability. Continuing advances in high-throughput sequencing technology may facilitate this. Despite this inter-individual variability, the present work identified a number of OTUs that showed significant changes in relative abundance after one and two weeks of experimental gingivitis. The analyses also identified OTUs that were negatively or positively correlated with bleeding on probing (BoP) scores. OTUs that decreased in

relative abundance over time and that were negatively correlated with BoP, were predominantly aerobic and facultatively anaerobic Gram-positive cocci and rods, including members of the genera *Actinomyces*, *Rothia*, and *Streptococcus*. It has been previously shown that *Streptococcus* spp., *Actinomyces* spp., and *Rothia* spp. are among the earliest colonisers of the tooth surface (Ritz, 1967, Diaz et al., 2006) and are prevalent in the mouths of healthy individuals (Aas et al., 2005, Bik et al., 2010) so it was unsurprising that members of these genera were abundant in health. An OTU identified as *Rothia dentocariosa* showed the most significant negative correlation with one- and two-week time points of experimental gingivitis, and with increased BoP scores. *R. dentocariosa* has previously been identified as a common constituent of the oral microbiome in health, particularly on tooth surfaces (Aas et al., 2005, Keijser et al., 2008, Bik et al., 2010) and the genus *Rothia* has been associated with oral health (Belda-Ferre et al., 2011, Griffen et al., 2012). Furthermore, a recent pyrosequencing study found that *R. dentocariosa* dominated the health-associated subgingival plaque communities analysed (Abusleme et al., 2013). The OTUs that increased in relative abundance as gingivitis developed and that were positively correlated with BoP scores were mostly Gram-negative taxa of the genera *Campylobacter*, *Fusobacterium*, *Lautropia, Leptotrichia, Porphyromonas, Selenomonas,* and *Tannerella*. Among those that have been previously cultivated, many were obligate anaerobes. These findings are largely in accordance with the observations of Theilade et al. (1966), who, in an early experimental gingivitis study, reported an increase in the proportion of Gram-negative cocci and rods as well as filaments, spirilla and spirochetes as gingivitis developed. The OTUs most strongly positively correlated with increased BoP scores included the unnamed phylotypes *Lachnospiraceae* [G-2] sp. HOT100 and *Lautropia* sp. HOTA94, as well as the

named previously cultured organisms *Fusobacterium nucleatum* subsp. *polymorphum* and *Prevotella oulorum*. An interesting observation of this study was the increased relative abundance in one- and two-week time points, and positive correlation with BoP, of an OTU identified as *Tannerella* sp. HOT286 (which includes oral clone BU063). This phylotype, a close relative of the putative periodontal pathogen *Tannerella forsythia*, was previously associated with periodontal health in a study using PCR to compare its prevalence in the 25% of a population with the most severe periodontitis to its prevalence in the 25% of the population with the best periodontal health (Leys et al., 2002). In that study, the relatively healthy subjects included some with pocket depths and attachment loss up to a maximum of 5 mm and, unlike the present study, the extent of gingival inflammation was not reported (Leys et al., 2002). Interestingly, *Tannerella* sp. oral clone BU063 was found to be more prevalent among individuals with gingivitis and necrotizing ulcerative gingivitis than in those with periodontitis in another study using fluorescent *in situ* hybridisation (Zuger et al., 2007). Furthermore, Huang et al. (2011) recently reported a significantly higher relative abundance of *Tannerella* sp. BU063 in the plaque of individuals with gingivitis than in healthy individuals.

The majority of OTUs that had a significantly higher relative abundance in chronic periodontitis patients than in healthy subjects were taxa that have been previously associated with periodontitis. However, one strongly associated OTU (*Leptotrichia* sp. HOTB57) may represent an additional taxon to add to this expanding list. *Porphyromonas gingivalis* had the greatest effect size among the associated OTUs and has been previously associated with periodontitis on the basis of both culture-dependent and -independent studies (Moore and Moore, 1994, Socransky et al., 1998, Kumar et al., 2003). Interestingly, *Prevotella denticola* was

among the other strongly periodontitis-associated OTUs. *P*. *denticola* was previously found to increase in incidence with increasing severity of periodontal disease based on culture (Wu et al., 1992), was detected at higher prevalence in periodontitis patients than healthy subjects using species-specific PCR (Kumar et al., 2003), and was strongly associated with chronic periodontitis in another recent pyrosequencing study (Griffen et al., 2012).

In addition to the detection of taxa that were significantly differentially represented between the cohorts and time points of experimental gingivitis, this work provided a detailed description of the taxonomic composition of dental plaque in periodontal health and disease. The phyla detected in these samples and their relative abundances are similar to those reported in previous studies (Huang et al., 2011, Abusleme et al., 2013), with plaque communities largely being dominated by the five phyla: *Firmicutes*; *Actinobacteria*; *Proteobacteria*; *Bacteroidetes*; and *Fusobacteria*. At lower taxonomic levels, previously identified common members of dental plaque such as *Streptococcus sanguinis* and the *Actinomyces naeslundii* group (Bik et al., 2010) were also highly represented. However, the frequent detection and high relative abundance of less well-known, or well-studied, organisms, including *Corynebacterium matruchotii*, *Leptotrichia buccalis* and *Leptotrichia hofstadii*, appears to be a new finding. This may be due to the limited number of samples, particularly from healthy individuals, that have been characterised at this sequencing depth and phylogenetic resolution, to date.

The cultural analysis undertaken in this chapter identified species of bacteria that were not detected by 454-pyrosequencing of the same samples, indicating that culture can still be a useful adjunct to molecular microbial community analyses, despite its associated biases. It should be noted, however, that a proportion of the

species detected only in culture could have been contaminants as they included common skin-associated staphylococci of the *S. epidermidis* group (Kong, 2011). The intra-isolate sequence variability of 16S rRNA copies observed in this study, especially among *Actinomyces* spp., was an intriguing finding. Previous work has shown that bacterial species may have multiple variable copies of the 16S rRNA gene within their genomes, with one study finding between 0 and 19 bases difference between copies (Coenye and Vandamme, 2003). However, the authors of that study concluded that this would not have a major influence on the classification of species as the percentage differences were usually below the thresholds commonly used to delineate species. In the present work, however, sufficient variability (>1.5% sequence dissimilarity) to result in assignment of partial 16S rRNA sequences to separate OTUs, among sequences of an *Actinomyces naeslundii* group isolate, was detected. This implies that there is the potential for over-estimation of species-level richness based on the clustering of OTUs at a sequence dissimilarity of 1.5%.

The volunteer group of healthy subjects were all clinical staff within the Dental Institute who were highly motivated and well informed in the practise of effective oral hygiene. The clinical condition in severe chronic periodontitis contributed a substantially different clinical environment for comparison, but the single samples collected from 20 patients with severe chronic periodontitis cannot be said to represent the entire population with periodontitis. The patients with periodontitis were significantly older than the healthy volunteers and this is a limitation of the current study. For ethical reasons it would not be appropriate to monitor changes in the microbiota whilst allowing irreversible destructive disease to progress over a number of years without intervention. The effects of patient age on the microbiota cannot be easily separated from the effect of the different microbial

habitat that develops as the patient ages and disease progresses. This study could not have been ethically designed to monitor the microbiota as gingivitis progressed to periodontitis or as periodontitis increased in severity with age. More extensive studies would be required to compare different types of periodontal disease, different levels of disease in different age groups and populations before a truly comprehensive description of the periodontal microbiome could be described with complete confidence. However, within these limitations, the current work has successfully applied deep sequencing technology to monitor short-term changes in the microbiota during the induction of reversible mild periodontal disease and contrasted it with the microbiota of a group of patients with severe and irreversible periodontal disease.

In conclusion, this chapter has revealed the presence of a highly rich bacterial biota in health-associated plaque and determined longitudinal shifts in bacterial community structure as plaque accumulates and gingivitis develops. The analyses both confirmed the association of a number of putative periodontal pathogens with chronic periodontitis and identified new health- and gingivitis-associated taxa. Further investigation of these taxa may lead to the development of novel therapies aiming to prevent the early stages of periodontal disease.

Chapter 3:

Bacterial antagonism of putative periodontal pathogens

Chapter 3: Bacterial antagonism of putative periodontal pathogens

3.1 Introduction

Gingivitis and chronic periodontitis are the result of the interaction between the diverse consortia of bacteria in dental plaque and the host's immune response (Darveau, 2010). Previous studies and the work presented in Chapter 2 have shown that the bacterial community membership and structure of plaque in periodontal health differs to that found in gingivitis and chronic periodontitis (Moore and Moore, 1994, Tanner et al., 1998, Huang et al., 2011). Moreover, chronic periodontitis has been associated with a number of bacterial species, most notably the 'red complex' group of putative periodontal pathogens: *P. gingivalis*, *T. forsythi*a and *T. denticola* (Socransky et al., 1998), although a number of other taxa have now been strongly associated with the disease including the Gram-positive organisms *F. alocis* and *Eubacterium saphenum* as well as the uncultured oral 'species' TM7 Human Oral Taxon 356 and *Synergistetes* Human Oral Taxon 362 (*Deferribacteres* clone D084) (Kumar et al., 2005, Griffen et al., 2012).

Currently, the primary means of preventing periodontal disease is through the use of oral hygiene methods that aim to reduce or remove plaque biofilms from the teeth, including tooth brushing, interdental cleaning and anti-microbial mouthrinses. However, many individuals do not practise oral hygiene to a standard and/or frequency sufficient to prevent gingivitis. Interestingly, recent work highlighting the potentially beneficial effects of the commensal oral microbiota, such as the denitrification of dietary nitrate to nitrite, suggest that frequent rigorous oral hygiene could inadvertently have negative consequences for the host (Kapil et al., 2013). For these reasons alternative strategies for the control and prevention of periodontal

disease are desirable. There has recently been an increased interest in developing approaches that aim to manipulate the composition of the microbiota such that it remains in a health-associated form. This may be possible through the use of oral probiotics or prebiotics. As discussed in Chapter 1 there has been a substantial amount of research into the potential for using probiotic organisms commonly used in the distal gut, such as *Lactobacillus* spp. and *Bifidobacterium* spp. as probiotics for the oral cavity. Both of these genera have, however been associated with caries (Nyvad et al., 2013), and it would clearly be desirable to use indigenous commensal oral bacteria for this purpose. The work described in Chapter 2 of this thesis, and other studies (Aas et al., 2005, Bik et al., 2010, Griffen et al., 2012, Abusleme et al., 2013), have now identified a number of bacterial species of the genera *Actinomyces*, *Corynebacterium*, *Rothia* and *Streptococcus* that are commonly present in dental plaque and associated with oral health. These bacteria may have the potential for use as oral probiotics through competition with putative pathogens for attachment sites (colonisation resistance) and/or by direct inhibition of their growth e.g. through the production of bacteriocins. For example, a bacteriocin-producing strain of *Streptococcus salivarius* (strain K12), which is a common species on the tongue dorsa of healthy individuals (Kazor et al., 2003), has been shown to be beneficial in the control of halitosis by inhibiting the growth of bacterial species that produce malodorous compounds (Burton et al., 2006). There is therefore the potential for oral bacterial strains that are antagonistic to the growth of putative periodontal pathogens to be used as oral probiotics for the treatment and prevention of periodontal disease.

There are a number of approaches that have been described for the *in vitro* detection of inhibitory interactions between bacteria, and these are typically variations of the commonly used ‘simultaneous antagonism’ and ‘deferred

antagonism' assays (Tagg et al., 1976). In the simultaneous antagonism assay the test and 'indicator' strains are grown together on an agar plate, with the latter usually as a confluent lawn. Importantly, the inhibitory agent, e.g. bacteriocin, must be released early in the growth of the test organism to allow detectable inhibition of the indicator organism (Tagg et al., 1976). The deferred antagonism method involves first growing the test strain as spots or streaks on the agar plate and then killing the test strain, before inoculating the indicator strain, either as an agar overlay or as streaks. This has the major advantage of allowing growth of the test and indicator strains under different conditions, if required. For example, antagonism of a strictly anaerobic organism by an obligate aerobe may be tested in this way.

The aim of this work was to test a panel of wild type oral bacterial isolates for their *in vitro* inhibitory activity against bacterial species that are associated with periodontal disease, in order to identify candidate oral probiotics.

3.2 Methods

The test strains were isolates from the culture analysis of plaque samples in experimental gingivitis (Chapter 2). In addition, the bacteriocin-producer (salivaricins A2 and B) *Streptococcus salivarius* K12 was obtained from BLIS Technologies Limited (New Zealand) and used as a positive control. The indicator strains were the type strains of species that have been associated with gingivitis or chronic periodontitis, in the present study and/or in previous studies, and are listed in Table 11. Test and indicator strains were grown and maintained on Blood Agar Base no. 2 (BA) + 5% v/v sterile defibrinated horse blood + 0.1% w/v calcium carbonate at 37°C in an anaerobic cabinet with an atmosphere of 80% N_2, 10% H_2 and 10% $C0_2$ or in air + 5% $C0_2$, in preparation for testing for inhibitory activity. For long-

term storage, isolates were suspended in Brain-Heart Infusion Broth (BHIB) + 10% glycerol and stored at -70°C.

Initial pilot work trialled the use of a simultaneous antagonism assay to detect inhibition of the indicator strains by *S. salivarius* K12. The test strain was inoculated onto the surface of BA + 0.1% w/v calcium carbonate using sterile toothpicks. Then, 100 µl of BHIB culture of the indicator strain, grown for 24-48 hours depending on the growth rate of the strain, was inoculated into 5 ml of molten BA + 0.1% calcium carbonate at 45°C. The inoculated molten medium was then poured over the surface of the inoculated test plates and incubated anaerobically at 37°C for 48 hours. Following incubation the plates were inspected for zones of inhibition in the lawn of growth over the area of the test strain. The assay did not demonstrate any detectable inhibition of *S. anginosus*, *F. nucleatum* or *S. moorei* by *S. salivarius* K12. Furthermore, problems were encountered relating to displacement of the *S. salivarius* K12 inoculum when agar was overlaid. The deferred antagonism assay described below was therefore selected for screening of the test isolates.

Table 11: Strains used as indicator organisms for the deferred antagonism assay.

Indicator strain (IS) no.	**Taxon**	**Strain number**
IS1	*Streptococcus anginosus*	CCUG 27298^{T}
IS2	*Fusobacterium nucleatum* subsp. *polymorphum*	ATCC 10953^{T}
IS3	*Solobacterium moorei*	JCM 10645^{T}
IS4	*Prevotella denticola*	CCUG 29542^{T}
IS5	*Porphyromonas gingivalis*	ATCC 33277^{T}
IS6	*Filifactor alocis*	ATCC 35896^{T}

3.2.1 *Deferred antagonism assay*

The assay used was based on that described and used previously (Tagg and Bannister, 1979, Burton et al., 2006) but with a number of modifications. Several colonies of the test strain (pre-grown on BA plates as described in the previous section) were harvested and suspended in 3 ml of sterile BHIB. A sterile cotton swab was then used to inoculate plates of Blood Agar Base no. 2 (LabM) + 5% v/v horse blood + 0.1% w/v calcium carbonate as a 1 cm-wide diametric streak. The plates were incubated at 37°C until good growth was visible, which was 24-72 hours depending on the growth rate of the test strain. Plates were incubated anaerobically for *Streptococcus* and in air + 5% $C0_2$ for *Rothia*, *Neisseria*, *Cardiobacterium*, *Corynebacterium*, and *Haemophilus* spp. Following incubation, a glass slide was used to remove visible growth of the test strain by scraping across the surface of the plate. The plate was then inverted over filter paper soaked with chloroform for 30 min and then exposed to the air for a further 30 minutes. Several colonies of the pre-grown indicator strains were each suspended into 3 ml of sterile BHIB and streaked across the plate at right angles to the original streak. Plates were then incubated anaerobically at 37°C for up to 72 hours and examined at 24-hour intervals for growth/growth inhibition. The experiments were performed on three occasions. The scoring system was as follows: no visible inhibition of the indicator strain - -; partial inhibition of the indicator strain over the test strain's original zone of growth - +; complete inhibition of the indicator strain over the test strain's original zone of growth - ++, complete inhibition of the indicator strain extending beyond the test strain's original zone of growth - +++.

3.2.2 *Agar well-diffusion assay*

Selected strains that demonstrated growth inhibition in the deferred antagonism assay were tested further in an agar well-diffusion assay. All experiments were performed in triplicate. First, 200 ml of sterile BHIB was inoculated with 200 µl of an 18-hour BHIB culture of the test organism diluted to an optical density (OD_{595}) of 0.1. The test organism was then incubated at 37°C anaerobically and growth was monitored by determination of the optical density (OD_{595}) of the culture at hourly intervals over a ten-hour period. In addition, 40 ml of the culture was removed at three time points during the lag, log and stationary growth phases, respectively, and centrifuged at 2365 x g for 15 mins. Following centrifugation, the supernatant was aspirated, its pH measured and then divided into two aliquots. The pH of one aliquot was adjusted to 7. The aliquots were then filtered using 0.2 µm filters (Millex® Millipore). Suspensions of indicator strains in BHIB (adjusted to an OD_{595} of 0.1) were used to inoculate Blood Agar Base no. 2 + 5% v/v horse blood plates to generate a confluent lawn of growth. Using the broad ends of sterile pipette tips, wells 7 mm in diameter were then cut into the agar. Next, 50 µl each of pH-adjusted and -unadjusted filtered culture supernatant from different time points, and sterile BHIB (negative control), were pipetted into separate wells on the plate. The supernatant was allowed to diffuse into the agar at room temperature before incubation at 37°C in an anaerobic atmosphere for 24-48 hrs. Plates were subsequently examined for zones of inhibition in the indicator lawn.

3.3 Results

Eighty isolates were tested for inhibitory activity against the indicator strains using the deferred antagonism assay. These isolates represented a range of different genera and species that are commonly present in the oral cavity in health (see Chapter 2) and were isolated from a number of different subjects recruited for the experimental gingivitis study. The results are shown in Table 12. Three isolates demonstrated inhibitory activity against one or more of the indicator strains. Examples of plates showing inhibitory activity are shown in Figures 30 and 31. Two isolates were identified as *Streptococcus cristatus*, and the other as *Streptococcus* sp. HOT071/HOTG62 by partial 16S rRNA gene sequencing. In addition, *S*. *salivarius* K12 was found to be inhibitory towards the growth of IS1 (*S*. *anginosus* CCUG 27298^{T}), IS2 (*S*. *moorei* JCM 10645 ^{T}) and IS6 (*F*. *alocis* ATCC 35896 ^{T}).

S. *salivarius* K12 and *S*. *cristatus* HS13BI05 were further tested using the agar well-diffusion assay. Cell-free supernatant samples from BHIB cultures of both strains at different time points (in lag, log and stationary growth phases), did not demonstrate any detectable growth inhibition of lawns of the indicators strains: *S*. *anginosus* and *S*. *moorei*. This was the case both for pH-adjusted and -unadjusted cell-free supernatant.

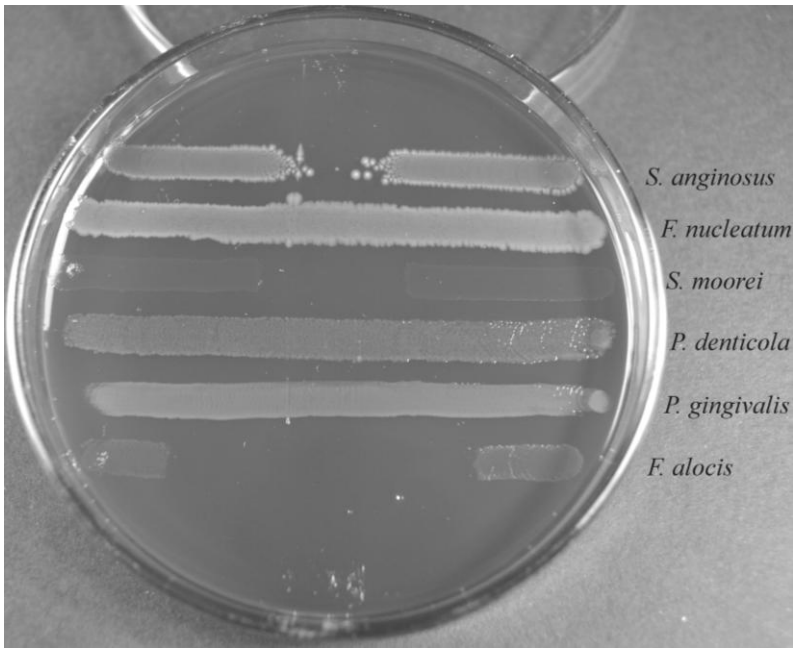

Figure 30: Deferred antagonism assay plate of *Streptococcus salivarius* K12.

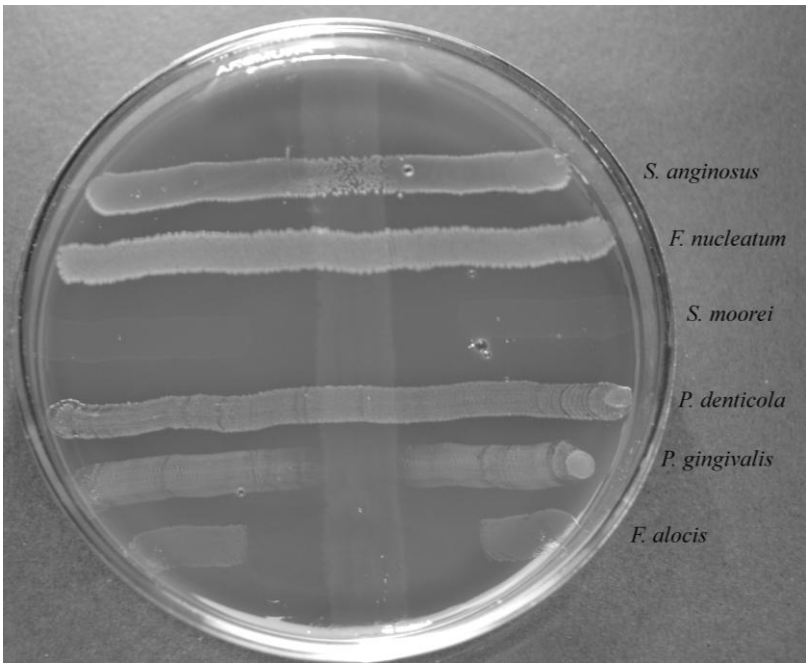

Figure 31: Deferred antagonism assay plate of *Streptococcus cristatus* HS13B_I05.

Table 12: Deferred antagonism inhibition of indicator organisms by oral bacterial isolates. The scoring system was as follows: no visible inhibition - -; partial inhibition over the test strain's original zone of growth - +; complete inhibition over the test strain's original zone of growth - ++, complete inhibition extending beyond the test strain's original zone of growth - +++. The indicator strains were *S. anginosus* - IS1; *F. nucleatum* - IS2; *S. moorei* – IS3; *P. denticola* - IS4; *P. gingivalis* - IS5; *F. alocis* - IS6.

Test strain isolate id / strain no.	Test strain identity (using 16S rRNA gene sequencing)	IS1	IS2	IS3	IS4	IS5	IS6
HS2B_I76	*Abiotrophia defectiva*	-	-	-	-	-	-
HS2B_I94	*Abiotrophia defectiva*	-	-	-	-	-	-
HS10B_I91	*Actinobaculum* sp. HOT183	-	-	-	-	-	-
HS14B_I76	*Actinobaculum* sp. HOT183	-	-	-	-	-	-
HS52W_I54	*Actinobaculum* sp. HOT183	-	-	-	-	-	-
HS13B_I94	*Actinomyces gerencseriae*	-	-	-	-	-	-
HS19B_I79	*Actinomyces gerencseriae*	-	-	-	-	-	-
HS7B_I83	*Actinomyces naeslundii* group	-	-	-	-	-	-
HS7B_I91	*Actinomyces naeslundii* group	-	-	-	-	-	-
HS13B_I28	*Cardiobacterium hominis*	-	-	-	-	-	-
HS19B_I78	*Cardiobacterium hominis*	-	-	-	-	-	-
HS5B_I12	*Cardiobacterium hominis*	-	-	-	-	-	-
HS13B_I26	*Corynebacterium durum*/HOTA22	-	-	-	-	-	-
HS19B_I18	*Corynebacterium durum*/HOTA22	-	-	-	-	-	-
HS3B_I84	*Granulicatella adiacens*	-	-	-	-	-	-
HS5B_I60	*Granulicatella adiacens*	-	-	-	-	-	-
HS10B_I01	*Haemophilus parainfluenzae*	-	-	-	-	-	-
HS13B_I89	*Haemophilus parainfluenzae*	-	-	-	-	-	-
HS1B_I09	*Neisseria elongata*	-	-	-	-	-	-
HS3B_I36	*Neisseria* sp. HOT009/014/015/016	-	-	-	-	-	-
HS2B_I14	*Rothia aeria*	-	-	-	-	-	-
HS2B_I31	*Rothia aeria*	-	-	-	-	-	-
HS16B_I04	*Rothia dentocariosa*	-	-	-	-	-	-
HS19B_I17	*Rothia dentocariosa*	-	-	-	-	-	-
HS7B_I05	*Rothia dentocariosa*	-	-	-	-	-	-
HS7B_I28	*Rothia dentocariosa*	-	-	-	-	-	-
HS7B_I36	*Rothia dentocariosa*	-	-	-	-	-	-
HS10B_I37	*Streptococcus cristatus*	-	-	-	-	-	-
HS13B_I05	*Streptococcus cristatus*	+	-	+++	-	+	+++
HS142W_I04	*Streptococcus cristatus*	-	-	-	-	-	-
HS14B_I12	*Streptococcus cristatus*	-	-	-	-	-	-
HS19B_I65	*Streptococcus cristatus*	++	-	+++	-	+	+++
HS1B_I88	*Streptococcus cristatus*	-	-	-	-	-	-
HS20B_I18	*Streptococcus cristatus*	-	-	-	-	-	-
HS20B_I19	*Streptococcus cristatus*	-	-	-	-	-	-
HS20B_I58	*Streptococcus cristatus*	-	-	-	-	-	-
HS5B_I42	*Streptococcus cristatus*	-	-	-	-	-	-
HS5B_I73	*Streptococcus cristatus*	-	-	-	-	-	-
HS3B_I12	*Streptococcus gordonii*	-	-	-	-	-	-
HS7B_I11	*Streptococcus gordonii*	-	-	-	-	-	-
HS7B_I41	*Streptococcus mitis* bv.2/HOTC56	-	-	-	-	-	-
HS7B_I51	*Streptococcus mitis* bv.2/HOTC56	-	-	-	-	-	-
HS12W_I70	*Streptococcus mitis* group	-	-	-	-	-	-
HS13B_I66	*Streptococcus mitis* group	-	-	-	-	-	-
HS13B_I68	*Streptococcus mitis* group	-	-	-	-	-	-
HS10B_I05	*Streptococcus mitis* group	-	-	-	-	-	-
HS10B_I74	*Streptococcus mitis* group	-	-	-	-	-	-

Test strain isolate id / strain no.	**Test strain identity (using 16S rRNA gene sequencing)**	**IS1**	**IS2**	**IS3**	**IS4**	**IS5**	**IS6**
HS13B_I24	*Streptococcus mitis* group	-	-	-	-	-	-
HS13B_I31	*Streptococcus mitis* group	-	-	-	-	-	-
HS14B_I15	*Streptococcus mitis* group	-	-	-	-	-	-
HS14B_I58	*Streptococcus mitis* group	-	-	-	-	-	-
HS16B_I32	*Streptococcus mitis* group	-	-	-	-	-	-
HS1B_93	*Streptococcus mitis* group	-	-	-	-	-	-
HS1B_I39	*Streptococcus mitis* group	-	-	-	-	-	-
HS1B_I59	*Streptococcus mitis* group	-	-	-	-	-	-
HS202W_I22	*Streptococcus mitis* group	-	-	-	-	-	-
HS202W_I60	*Streptococcus mitis* group	-	-	-	-	-	-
HS2B_I19	*Streptococcus mitis* group	-	-	-	-	-	-
HS2B_I64	*Streptococcus mitis* group	-	-	-	-	-	-
HS2B_I69	*Streptococcus mitis* group	-	-	-	-	-	-
HS2B_I87	*Streptococcus mitis* group	-	-	-	-	-	-
HS3B_I31	*Streptococcus mitis* group	-	-	-	-	-	-
HS5B_I62	*Streptococcus mitis* group	-	-	-	-	-	-
HS5B_I89	*Streptococcus mitis* group	-	-	-	-	-	-
HS72W_I11	*Streptococcus mitis* group	-	-	-	-	-	-
HS7B_I01	*Streptococcus mitis* group	-	-	-	-	-	-
HS7B_I02	*Streptococcus mitis* group	-	-	-	-	-	-
HS7B_I09	*Streptococcus mitis* group	-	-	-	-	-	-
HS14B_I34	*Streptococcus oralis*/HOTC08/HOT061/HOTE24	-	-	-	-	-	-
HS14B_I30	*Streptococcus oralis*/HOTC08/HOT061/HOTE24	-	-	-	-	-	-
K12	*Streptococcus salivarius*	++	-	+++	-	-	+++
HS1B_I04	*Streptococcus sanguinis*	-	-	-	-	-	-
HS1B_I08	*Streptococcus sanguinis*	-	-	-	-	-	-
HS2B_I06	*Streptococcus sanguinis*	-	-	-	-	-	-
HS2B_I07	*Streptococcus sanguinis*	-	-	-	-	-	-
HS5B_I06	*Streptococcus sanguinis*	-	-	-	-	-	-
HS5B_I07	*Streptococcus sanguinis*	-	-	-	-	-	-
HS72W_I18	*Streptococcus* sp. HOT071/HOTG62	-	-	++	-	-	-
HS72W_I31	*Streptococcus* sp. HOT071/HOTG62	-	-	-	-	-	-
HS102W_I89	*Veillonella parvula*/*dispar*	-	-	-	-	-	-
HS13B_I62	*Veillonella parvula*/*dispar*	-	-	-	-	-	-

3.4 Discussion

This is one of relatively few studies investigating the antagonism of putative periodontal pathogens by bacteria commonly isolated from dental plaque (Hillman et al., 1985a, van Essche et al., 2013), although bacteriocin-like inhibitory substances (BLIS) produced by strains of *S*. *salivarius* have been isolated and characterised (Tagg, 2004, Burton et al., 2006, Hyink et al., 2007, Masdea et al., 2012). In the present study, *S*. *salivarius* K12 was found to inhibit the growth of indicator strains of the Gram-positive taxa *S*. *anginosus*, *S*. *moorei* and *F*. *alocis,* confirming previous work showing that this strain inhibits *S*. *moorei* and *S*. *anginosus* strains in deferred antagonism assays (Burton et al., 2006, Masdea et al., 2012). Inhibition of *F*. *alocis* by *S*. *salivarius* K12, however, has not been previously reported. The ability of *S*. *salivarius* K12 to inhibit the growth of this putative periodontal pathogen, and others, is suggestive of a potential application for this strain in the prevention and control of periodontal disease. However, *S*. *salivarius* was detected at a low prevalence and abundance in dental plaque in health, gingivitis, and chronic periodontitis, using 454-pyrosequencing in Chapter 2 (<100 sequences in the total dataset, see appendix) and the organism was not isolated in culture. Other studies have shown that *S*. *salivarius* is more commonly present in saliva and on mucosal surfaces than on the tooth surface or in subgingival dental plaque (Aas et al., 2005). As dental plaque is of primary aetiological importance in gingivitis and chronic periodontitis, the efficacy of the probiotic organism may be enhanced if the species is already naturally well adapted to colonise and multiply within this ecological niche. Introduction of strain(s) of such species capable of producing bacteriocins, or other substances, inhibitory to the growth of putative periodontal pathogens could modulate the

composition of the plaque community such that it remains compatible with periodontal health.

In the present study, growth antagonism of the indicator strains was detected in a relatively small proportion of the test isolates (3.75% of the isolates tested) and was limited to members of the genus *Streptococcus*, although this genus constituted the majority of the isolates tested. Further screening of a larger panel of isolates with greater representation of other genera may reveal other species/strains with inhibitory activity. In a recent study, van Essche et al. (2013) used an agar overlay assay to screen 1750 individual bacterial colonies isolated from subgingival dental plaque for potential growth inhibition of the periodontitis-associated species: *P. gingivalis*, *P. intermedia*, *F. nucleatum* subsp. *vincentii* and *A. actinomycetemcomitans*. The authors found that 72 (4.2%) of the colonies tested demonstrated inhibitory activity against one or more of the indicator species: similar to the proportion of inhibitory isolates found in the current study. Further testing with an agar well-diffusion assay confirmed inhibitory activity in 48 of these, the most strongly inhibitory were identified (a total of 19) and found to be predominantly *Streptococcus* spp. and *Actinomyces* spp. As inhibitory activity was only detected when using overnight broth culture suspensions and not cell-free supernatant (from the overnight broth cultures) of these isolates, the authors concluded that the antagonism was the result of competition for nutrients or possible acid production. As the overnight culture suspensions that showed activity were tested without killing or removal of viable cells, the test strain would have likely grown in the agar surrounding the wells. Therefore, inhibitors may have been produced at this stage rather than whilst growing in the broth.

Deferred antagonism testing in the present study revealed inhibitory activity (against the growth of one or more of the indicator strains) of two isolates of *S. cristatus* from different individuals, and one isolate of the un-named organism *Streptococcus* sp. HOT071/HOTG62. The indicator strains used in this study were selected to be representative of both Gram-positive and Gram-negative species, all of which have previously been associated with gingivitis and/or chronic periodontitis (Socransky et al., 1998, Colombo et al., 2009, Huang et al., 2011, Griffen et al., 2012). The two inhibitory *S. cristatus* isolates exhibited a similar inhibitory pattern to one another in terms of the organisms targeted. Those species that were susceptible (with the notable exception of *P. gingivalis*) were Gram-positive species, all belonging to the phylum *Firmicutes*. Inhibition of some of the indicator strains beyond the original zone of growth of the test strains indicated that the inhibitory agent(s) were able to diffuse through the solid media. These observations suggest that the observed inhibition may have been due to BLIS production: bacteriocins of Gram-positive bacteria are predominantly low molecular weight peptides, readily diffusible in agar media, that usually target other, often closely-related, Gram-positive taxa (although some have also been found to inhibit Gram-negative species) (Jack et al., 1995, Nes et al., 2007). Purification and characterisation of the compound(s) responsible, however, would be necessary to draw this conclusion with certainty. As reviewed in Chapter 1, antagonism of bacteria by a particular species may be the result of the production of other substances such as acids, hydrogen peroxide or inhibitory enzymes. The inclusion of calcium carbonate as a buffering agent against acid-induced changes in pH in the present study reduced the likelihood that the observed inhibitory activity was a result of acid production by the test strains. This is further supported by the observation that other isolates of the same

species (*S. cristatus* and *Streptococcus* sp. HOT071), which could also be expected to produce lactic acid as a product of carbohydrate fermentation, did not exhibit any detectable inhibition. The anaerobic incubation used for the *Streptococcus* spp. test strains precluded hydrogen peroxide production-mediated inhibition of the indicator strains. This is because hydrogen peroxide synthesis is down-regulated in the absence of oxygen (Barnard and Stinson, 1999).

The lack of detectable inhibition of indicator strains in the agar well-diffusion assay using cell-free supernatants of isolates found to have inhibitory activity in the deferred antagonism assay (including *S. salivarius* K12), was perhaps surprising. This may have been a result of the different media used and the fact that, unlike in streak cultures on agar plates, cells in a standard liquid broth culture do not grow as a biofilm. Both of these factors could have had a possible effect on the expression of the inhibitory substances, resulting in a sub-inhibitory yield and concentration when tested in the agar well-diffusion assay. Previous studies of bacteriocin-producing streptococci have also reported difficulties in recovering these inhibitory substances after growth in liquid media (Dempster and Tagg, 1982, Wescombe and Tagg, 2003). Wescombe and Tagg (2003) reported that all attempts to detect production of the *Streptococcus pyogenes* bacteriocin streptin by a known producer strain in liquid cultures were unsuccessful. However, the investigators successfully used a biphasic culture method to detect inhibitory activity and purify the bacteriocin from the liquid phase culture supernatant. In the case of *S. salivarius* K12, purification of the lantibiotics: salivaricins A2 and B has previously been achieved by freeze-thaw extraction from lawn cultures on agar (Hyink et al., 2007). Future work on the inhibitory strains found in this study could utilise one of these methods in attempts to purify the inhibitory compound(s).

Bacteriocin-like inhibitory activity by *S*. *cristatus* has not been reported previously, although interestingly Wue and Zie (2010) reported the inhibition of *P*. *gingivalis in vitro* biofilm formation through the *S*. *cristatus* surface protein arginine deiminase (*arcA*). More recently, the same group showed that *S*. *cristatus arcA* was able to attenuate *P*. *gingivalis* colonisation of the oral cavities of mice and reduce subsequent *P*. *gingivalis*-induced alveolar bone loss (Xie et al., 2012). Since, *S*. *cristatus* and *P*. *gingivalis* cells were never in direct contact for the assay used in this study it seems unlikely that this was the mechanism responsible for the inhibition of *P*. *gingivalis*. It would be useful to perform further testing to determine if other chronic periodontitis-associated organisms, as well as other common commensal oral species, are susceptible to inhibition by the *S*. *cristatus* strains from this study. In Chapter 2, *S*. *cristatus* was identified as a common member of dental plaque in health, comprising up to 11.4% of the total microbiota in one subject. In addition, an OTU identified as *S*. *cristatus* had a significantly higher relative abundance in healthy subjects at baseline than in patients with chronic periodontitis (as determined using LEfSe). Intriguingly, *S*. *cristatus* has been shown to modulate the host's inflammatory immune response to *Fusobacterium nucleatum* subsp. *polymorphum in vitro* through inhibition of NF-kB activation and suppression of IL-8 (Zhang and Rudney, 2011). This anti-inflammatory effect may be an additional benefit to the potential use of BLIS-producing strains of *S*. *cristatus* as periodontal probiotics. Further characterisation of the strains found to be inhibitory in this work, such as determination of their susceptibility to antibiotics, is warranted in order to assess their suitability and safety as potential probiotic organisms.

Chapter 4:

Phylogenetic analysis of oral *Neisseria*

Chapter 4: Phylogenetic analysis of oral *Neisseria*

4.1 Introduction

Neisseria are common members of the human oral microbiome. Relatively little is known, however, about their role in oral health and disease. One reason for this could be that identification of oral *Neisseria* species using conventional phenotypic methods and by 16S rRNA gene sequencing is unreliable (Hanage et al., 2005, Bennett et al., 2012). The taxonomy of the group is unreliable in that there are questions regarding the validity of a number of the currently named species. Many of the species found in the oral cavity were described principally on the basis of their colony morphology and patterns of acid production from different carbohydrates. However, considerable variability in these phenotypes has been recorded (Knapp, 1988), among different strains of the same species, and for the same strains in different studies, bringing into question their identity. Recent comparisons of genomic sequences of *Neisseria* reference strains have suggested that some of the early named species should be consolidated and re-named (Bennett et al., 2012). For instance, it was proposed that *N. flavescens* is sufficiently closely related to *N. subflava* to be considered a variant of this species. Moreover, the authors noted that, based on the clustering of isolates in phylogenetic trees, some of the named *Neisseria* strains held in culture collections had been incorrectly classified.

Although sequence analysis of the 16S rRNA gene is undoubtedly a useful tool for bacterial identification, it cannot differentiate between many oral *Neisseria* species. Indeed, many of the *Neisseria* isolates recovered from the experimental gingivitis cohort in Chapter 2 could not be assigned to a single species and so a list of the possible alternatives was given. Other molecular means of identification, such as

Multi-Locus Sequence Analysis (MLSA) of housekeeping genes, including ribosomal protein gene sequences, may be viable alternatives. Studies using these approaches have found that they offer a greater resolution with which to discriminate between closely related taxa than 16S rRNA gene analysis, due to greater sequence variation at the loci used (Martens et al., 2008, Bennett et al., 2012, Jolley et al., 2012). For example, using a phylogenetic tree constructed from 53 ribosomal protein genes, Jolley et al. (2012) were able to resolve the closely related streptococci: *S. pneumoniae* and *S. mitis*, which share >99% 16S rRNA gene sequence identity (Do et al., 2009). In addition, the phylogenies based on individual ribosomal protein genes were highly congruent with each other, even for naturally competent groups that frequently engage in horizontal gene transfer, such as *Neisseria* spp. It was therefore posited that for species-level identification, sequence analysis of a single ribosomal protein gene might be sufficient (Jolley et al., 2012). PCR and sequencing of the 50S ribosomal protein L6 gene (*rplF*), one of the ribosomal protein genes used in rMLST, was recently used as a means of identifying *N. meningitidis* in pharyngeal swab samples in order to survey meningococcal carriage in the African meningitis belt (Ali et al., 2013). There have been no reported attempts to use MLSA or ribosomal MLST to examine the phylogenetic relationships of a collection of oral *Neisseria* isolates in conjunction with reference strains from culture collections. Such an investigation would be useful in order to determine if phylogenetic groups/clusters consistent with currently described taxa can be delineated and whether or not modifications to the existing taxonomy are required.

This work aimed to evaluate the use of alternative phylogenetic methods for the identification of oral *Neisseria* spp., including an MLSA scheme and analysis of the 50S ribosomal protein gene L6 (*rplF*).

4.2 Methods

4.2.1 *Selection of reference strains and oral isolates*

Following review of the relevant literature and of the Human Oral Microbiome Database (HOMD) appropriate reference strains of *Neisseria* species were selected on the basis of previous detection in the human oral cavity and by their inclusion in the current List of Prokaryotic Names with Standing in Nomenclature (LPSN) (Euzeby, 1997) at the time of planning, and are shown in Table 13. The type strain of the pathogen *N*. *meningitidis* was also obtained and included in the analysis. Strains were purchased from the Culture Collection, University of Göteborg (CCUG), Sweden. In addition, *Neisseria* isolates that were recovered from dental plaque samples taken from the experimental gingivitis cohort (Chapter 2) and oral isolates from previous work were included in the analyses. All strains were subjected to 16S rRNA gene PCR and sequencing using the method described for the identification of isolates in Chapter 2.

All strains/isolates were grown and maintained on Blood Agar Base no.2 (LabM) with 5% v/v sterile defibrinated horse blood and incubated in air + 5% $C0_2$ at 37˚C. For long-term storage, strains were frozen in Brain-Heart Infusion Broth (BHIB) with 10% v/v glycerol at -70˚C.

Table 13: *Neisseria* reference strains included in this study

Species	Strain no.
Neisseria flavescens	CCUG 17913^T
Neisseria elongata subsp. *elongata*	CCUG 2130A^T
Neisseria weaveri	CCUG 4007^T
Neisseria perflava	CCUG 17915^T
Neisseria polysaccharea	CCUG 18030^T
Neisseria sicca	CCUG 23929^T
Neisseria subflava	CCUG 23930^T
Neisseria mucosa	CCUG 26877^T
Neisseria bacilliformis	CCUG 50858^T
Neisseria meningitidis	CCUG 3269^T
Neisseria flava	CCUG 24961^T

4.2.2 *PCR and sequencing*

Primers described previously for the *N. meningitidis* Multi-Locus Sequence Typing (MLST) scheme (Maiden et al., 1998) were obtained for PCR and sequencing of the following 'housekeeping' genes: *abcZ* (putative ABC transporter), *adk* (adenylate kinase), *aroE* (shikimate dehydrogenase), *fumC* (fumarate hydratase), *gdh* (glucose-6-phosphate dehydrogenase), *pdhC* (pyruvate dehydrogenase subunit) and *pgm* (phosphoglucomutase), and are detailed in Table 14. Some additional variants of these primers from previous MLST work on commensal *Neisseria* were suggested and used, following personal communication (Dr. Julia Bennett, University of Oxford). In addition, previously designed primers (unpublished data, Dr. Julia Bennett) were obtained for the amplification and sequencing of a 413 bp region of the 50S ribosomal protein L6 gene *rplF*. Using the same reagent mix as described for the 16S rRNA gene PCR in Chapter 2, and using the 'touch PCR' protocol, PCR

amplification of the seven MLST 'housekeeping' genes and the *rplF* gene of strains was performed under the following conditions: 94˚C for 2 mins, followed by 30 cycles of 94˚C for 1 min, 50-60˚C for 1 min, 72˚C for 2 mins and a final extension of 72˚C for 2 mins. The annealing temperatures were determined for each set of primers accordingly, and initially performed at 5˚C below the lowest Tm of each primer pair. *N. meningitidis* was included as a positive control in each set of PCR reactions along with a negative (no template) control. PCR products were visualised by electrophoresis on a 1% agarose gel and assessed for the presence of single bands of the correct size. Where amplification was initially unsuccessful, reference genome sequences from Genbank, for the species: *N. flavescens, N. elongata* subsp. *glycolytica, N. mucosa, N. polysaccharea, N. sicca, N. subflava* and *N. meningitidis,* were downloaded and aligned with ClustalW in BioEdit. Alignments were visually inspected and the regions of sequence used for analysis in the *Neisseria* MLST scheme were determined. PCR primers were then re-designed to anneal to the most conserved sequences in the alignments that would amplify regions of approximately 900-1000 bp (covering the region targeted by the sequencing primers). Due to the degree of variability between sequences, even in relatively conserved regions, multiple variants of each forward and reverse primer were designed for each gene, some of which included degenerate nucleotides. Figure 32 shows an example of the newly designed forward primer aroE-NP1B-F and the previously described forward primer aroE-P1B aligned with the target sequence. For amplification of the *rplF* gene, modifications were made to the primers for some species by alignment and visual inspection of reference sequences available in GenBank. After successful amplification, amplicons were treated with ExoSAP-IT and sequenced using the Big Dye® Terminator v3.1 cycle sequencing kit using the appropriate forward and

reverse primers as described in Chapter 2. ABI sequence chromatograms were inspected in the Sequence Scanner software and imported into BioEdit in the FASTA format for further analysis. Consensus sequences were constructed from the forward and reverse sequences using the contig assembly program (CAP)(Huang, 1992). The sequences for each gene were subsequently aligned by ClustalW and phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei, 1987) with Jukes Cantor correction in the Molecular Evolutionary Genetics Analysis (MEGA) version 4 software.

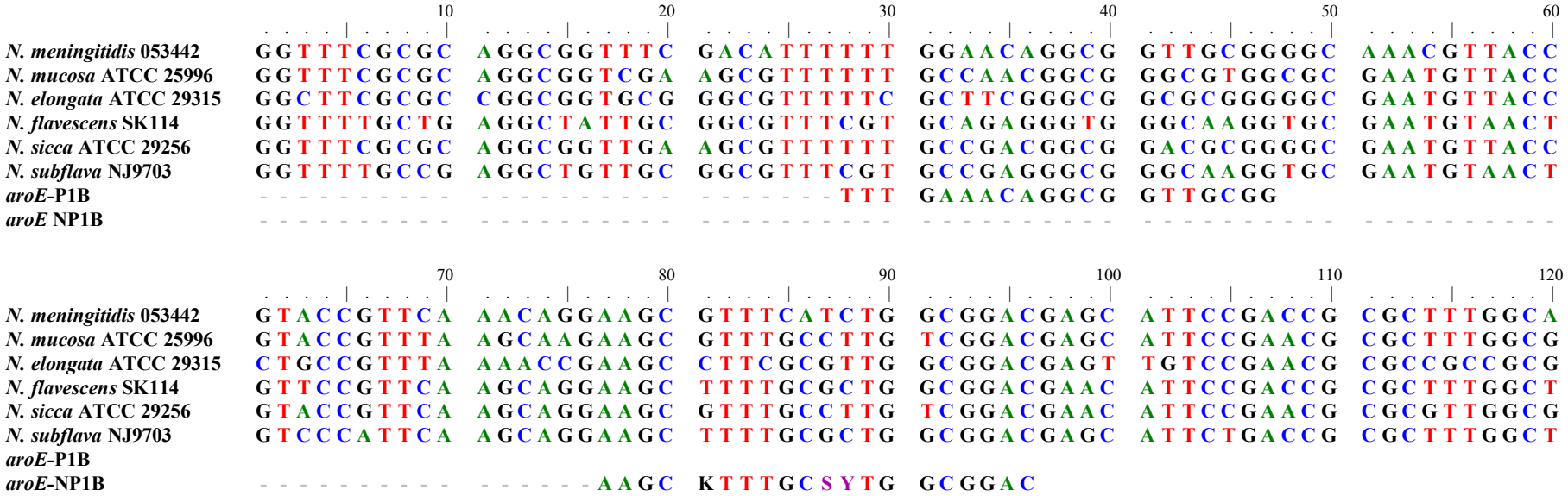

Figure 32: Region of an alignment of the *aroE* gene for *Neisseria* reference strains (downloaded from GenBank) used for forward primer design. Sequences are shown in the 5' – 3' orientation from left to right. The original previously described forward primer is named aroE-P1B and shows numerous mismatches to the target sequences. The newly designed primer with degenerate bases is aroE-NP1B.

Table 14: Previously designed PCR and sequencing primers used in this study

PCR primer	Gene target	Sequence (5'-3')
abcZ-P1C	*abcZ*	TGTTCCGCTTCGACTGCCAAC
abcZ-P2C	*abcZ*	TCCCCGTCGTAAAAAACAATC
adk-P1B	*adk*	CCAAGCCGTGTAGAATCGTAAACC
adk-P2B	*adk*	TGCCCAATGCGCCCAATAC
C-adk-P1	*adk*	CCKCAGATYTCYACAGGCGA
C-adk-P2	*adk*	AATAYTTCKGCTTTCACGGC
aroE-P1B	*aroE*	TTTGAAACAGGCGGTTGCGG
aroE-P2B	*aroE*	CAGCGGTAATCCAGTGCGAC
fumC-P1B	*fumC*	TCCCCGCCGTAAAAGCCCTG
fumC-P2B	*fumC*	GCCCGTCAGCAAGCCCAAC
fumC-PFW	*fumC*	GGCYTRCCRTTTGTCAG
fumC-PRW	*fumC*	TKRTARGCGGTTTTGGCG
gdh-P1B	*gdh*	CTGCCCCCGGGGTTTTCATCT
gdh-P2B	*gdh*	TGTTGCGCGTTATTTCAAAGAAGG
pdhC-PB1	*pdhC*	CCGGCCGTACGACGCTGAAC
pdhC-PB2	*pdhC*	GATGTCGGAATGGGGCAAACA
pgm-PB1	*pgm*	CGCCTCAAAACGCAACACCAG
pgm-PB2	*pgm*	ACGGCACTTTCCCCAACCACC
rplF-F	*rplF*	CAGTGACTGTTCCCGCTGGTGT
rplF-R	*rplF*	AGGYTCAGGAGKWCGGAAHG

Table 15: Modified PCR and sequencing primers designed and used in this study

PCR/sequencing primer	Gene target	Sequence (5'-3')
abcZ-NP1-F	*abcZ*	TCGCCGGTGTGCAAAAGCTCG
abcZ-NP1-R	*abcZ*	GCGCTGCGGAACTGGTCGAA
abcZ-NP2-F	*abcZ*	TCGCCGGYGTRCARAAGCTCG
abcZ-NP2-R	*abcZ*	GCGCTRCGRAAYTGGTCGAA

PCR/sequencing primer	**Gene target**	**Sequence (5'-3')**
abcZ-NPE-F	*abcZ*	TCGCCGGCGTGCAGAAACCCG
abcZ-NPE-R	*abcZ*	GCGCTGCGGAATTGGTCGAAA
abcZ-S1A	*abcZ*	AATCGTTTATGTACCGCAGR
abcZ-S2	*abcZ*	GAGAACGAGCCGGGATAGGA
adk-S1A	*adk*	AGGCWGGCACGCCCTTGG
adk-S2	*adk*	CAATACTTCGGCTTTCACGG
aroE-NP1-F	*aroE*	AAGCTTTTGCGCTGGCGGAC
aroE-NP1B-F	*aroE*	AAGCKTTTGCSYTGGCGGAC
aroE-NP1C-F	*aroE*	AWGCYTTYGCGTTGGCGGAC
aroE-NP2-R	*aroE*	TGGCTGATGGTCGAACCGCC
aroE-NP2B-R	*aroE*	TGYCTGATGGTCGAYCCGCC
aroE-NP2C-R	*aroE*	TGACTGATGGTCGAGCCGCC
aroE-S1A	*aroE*	GCGGTCAAYACGCTGRTK
aroE-S2	*aroE*	ATGATGTTGCCGTACACATA
fumC-NP1-F	*fumC*	GCGAACCGCGCCAACGAAAT
fumC-NP1B-F	*fumC*	GCSAWCCGCGCCAACGAAAT
fumC-NP1C-F	*fumC*	GCCATGCGCGATGGTRACGT
fumC-NP2-R	*fumC*	GATGGCGGTTTCGCGCAACG
fumC-NP2B-R	*fumC*	YACGGCGGTTTCGCGCAYCG
fumC-NP2C-R	*fumC*	CGCGCAGACCCAGCGCAGCC
fumC-S1	*fumC*	TCCGGCTTGCCGTTTGTCAG
fumC-S2	*fumC*	TTGTAGGCGGTTTTGGCGAC
gdh-NP1-F	*gdh*	ACGTGCGCGTGGTTTTGGAA
gdh-NP1B-F	*gdh*	GCGGTGCGGCTGCCTTCAAA
gdh-NP1C-F	*gdh*	RCGKGYTTGGCTGTTTTSAAA
gdh-NP2-R	*gdh*	TACTGGTCTTCGTGCCACTTG
gdh-NP2B-R	*gdh*	TATTGCTCTTCGTGCCACTTG
gdh-NP2C-R	*gdh*	GCGCGCAGCTTCAGGACCC
gdh-S3	*gdh*	CCTTGGCAAAGAAAGCCTGC
gdh-S4C	*gdh*	RCGCACGGATTCATRYGG

PCR/sequencing primer	**Gene target**	**Sequence (5'-3')**
L-gdh-P3	*gdh*	GTTTTGCCAACGTGATGTTC
L-gdh-P4	*gdh*	TATAGAGGCGGACGGATTCGGT
pdhC-NP1-F	*pdhC*	TTCCAACGCGTCGGCGACTT
pdhC-NP1B-F	*pdhC*	TTCCAACGYGYCGGYGACTT
pdhC-NP1C-F	*pdhC*	TTTTCATGYACCGCCGACTT
pdhC-NP2-R	*pdhC*	ACCACCATGGCTTTCGTCCGC
pdhC-NP2B-R	*pdhC*	TCTTTGCTGACTTTGCCTTGT
pdhC-S1	*pdhC*	TCTACTACATCACCCTGATG
pdhC-S2	*pdhC*	ATCGGCTTTGATGCCGTATTT
pdhC-seqF	*pdhC*	GTGTTCTACTACATCACCCTGAT
pdhC-seqR	*pdhC*	CAATGGCTTGTTGAACGG
pgm-NP1-F	*pgm*	GCTCGCAGGCGAAGCCATTCA
pgm-NP1B-F	*pgm*	KRCCGYCGTCGAAGCCRAACC
pgm-NP1C-F	*pgm*	TGCCGTCGTCGAAGCCGAACC
pgm-NP2-R	*pgm*	TTGGAAGCACGCATCAGGCCG
pgm-NP2B-R	*pgm*	GGSYWCACCCGCATCYCACTC
pgm-NP2C-R	*pgm*	AACATCCGCGCCGTCGCCGGTC
pgm-S1	*pgm*	CGGCGATGCCGACCGCTTGG
pgm-S2A	*pgm*	GGTGATGATTTCGGTYGCRCC
rplF-F-wea	*rplF*	CAGTGACTGTTCCTGCTGGTGT
rplF-F-020	*rplF*	CAGTATTTGTTCCTGCTGGGGT
rplF-F-bac	*rplF*	CTGTAAGCGTTCCTGAAGGTGT
rplF-R-wea	*rplF*	AGGCTCTGGAGAGCGAAATG
rplF-R-020	*rplF*	AGGTTCTGGGGAACGAAAAG
rplF-R-bac	*rplF*	AGGCTCAGGAGAACGAAAGG

4.3 Results

4.3.1 *16S rRNA gene sequence analysis*

High quality partial 16S rRNA gene sequences were obtained for all of the reference strains. BLAST analysis of these sequences in HOMD confirmed the identity of the strains, at a threshold of >98.5% sequence identity for three of the strains, which were identified as *N. elongata*, *N. bacilliformis*, and *N. weaveri*. The sequences of the *N. flavescens*, *N. meningitidis*, *N. mucosa*, *N. perflava*, *N. polysaccharea*, *N. sicca*, and *N. subflava* reference strains, all gave more than one match above the 98.5% sequence identity threshold (Table 16). The *N. flava* strain was identified as a member of the *Moraxella* genus, indicating a likely mislabelling or misidentification, and was excluded from further analyses. The oral isolates included in the analyses were assigned to the following phylotypes/groups by BLAST analysis of 16S rRNA gene sequences in HOMD: *N. elongata* (31 isolates), *N. flavescens*/*subflava* (24), *N. oralis*/HOT009/HOT15/HOT016 (15), *N. flava*/*mucosa*/*pharyngis*/*sicca* (14), *N. bacilliformis* (3), *Neisseria* sp. HOT020 (3), *Neisseria* sp. HOT018 (1). A phylogenetic tree constructed from the 16S rRNA sequences of reference type strains and of the oral isolates is shown in Figure 33.

Table 16: 16S rRNA gene identifications of *Neisseria* reference strains using BLAST in HOMD. All of the sequences used for BLAST were 402 bases in length.

Reference strain identity as given by the CCUG	**16S rRNA gene-based classification using HOMD (% sequence identity (excluding gaps and non-AGCTU))**
Neisseria flavescens	*Neisseria flavescens* (100%) / *subflava* (99.5%)
Neisseria elongata subsp. *elongata*	*Neisseria elongata* (99.8%)
Neisseria weaveri	*Neisseria weaveri* (100%)
Neisseria perflava	*Neisseria flavescens* (99.8%) / *subflava* (99.8%)
Neisseria polysaccharea	*Neisseria polysaccharea* (100%) / *meningitidis* (99.8%)
Neisseria sicca	*Neisseria pharyngis* (99.8%) / *flava* (99.8%) / *sicca* (99.5%) / *mucosa* (99.5%)
Neisseria subflava	*Neisseria flavescens* (99.8%) / *subflava* (99.8%)
Neisseria mucosa	*Neisseria mucosa* (99.8%) / *sicca* (99.8%) / *pharyngis* (99.5%) / *flava* (99.5%)
Neisseria bacilliformis	*Neisseria bacilliformis* (99.5%)
Neisseria meningitidis	*Neisseria meningitidis* (99.5%) / *polysaccharea* (99.3%)
Neisseria flava	*Moraxella caviae* (99%)

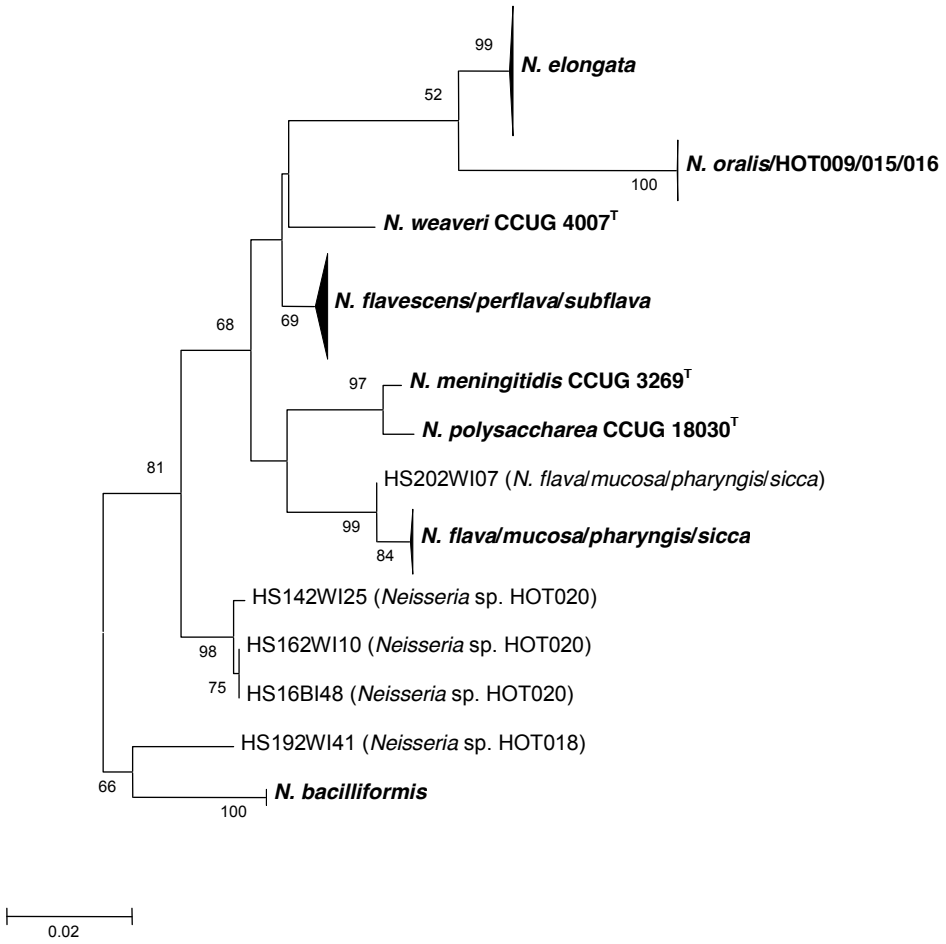

Figure 33: Phylogenetic tree based on an alignment of partial 16S rRNA gene sequences of *Neisseria* reference strains and oral isolates. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

4.3.2 *Multi-Locus Sequence Analysis (MLSA)*

The MLSA scheme was initially developed using the reference type strains detailed in Table 12. Of the primers previously described for *N. meningitidis*, only primers C-adk-P1 and P2, targeting the *adk* gene, successfully amplified genes in all species tested. The high heterogeneity of gene sequences required the design of several new sets of primers for PCR amplification and sequencing of the MLSA loci. Re-designed primers for the remaining genes resulted in amplification of all genes for seven of the reference strains (Table 17). Sequences for all genes were not obtained for the strains of *N. bacilliformis*, *N. elongata* subsp. *elongata* and *N. weaveri*. The MLSA scheme was used for 16 of the oral isolates. However, difficulties were again encountered in amplifying the genes, even with re-designed primers. In particular, several genes of taxa provisionally identified (by 16S rRNA gene sequence analysis) as *N. flavescens*/*subflava*, *N. elongata*, and *N. flava*/*mucosa*/*pharyngis*/*sicca* could not be amplified and sequenced.

Phylogenetic trees based on sequences for the seven individual genes are shown in Figures 34-40. Visual inspection of the trees shows that they were largely incongruent, with certain isolates clustering with different groups, depending on the gene analysed. For example, the *N. polysaccharea* type strain which is most closely related to the *N. meningitidis* type strain by 16S rRNA gene sequencing, clustered with *N. meningitidis* in the *gdh* and *pdhC* trees (Figures 38 and 39) but was placed in other branches (mostly with *N. flavescens*/*subflava* isolates) in the remaining trees. However, there were some isolates that clustered more consistently. In particular, isolates identified as *N. flavescens*/*subflava* by 16S rRNA gene sequencing, along with the type strains of *N. flavescens*, *N. subflava* and *N. perflava* frequently grouped together. In the instances where sequences were obtained for *N. bacilliformis* (for the

adk and *aroE* genes) and *N*. *weaveri* (for the *adk* gene), they appeared to be distinct from the other *Neisseria* spp., being placed in separate branches of the trees. The tree derived from concatenated sequences from all seven genes is shown for the 10 strains (Figure 41) for which the full set of sequences could be obtained. The clustering of the strains appears to be largely consistent with the 16S rRNA gene tree. One cluster consisted of the *N*. *flavescens*, *N*. *perflava* and *N*. *subflava* type strains along with two oral isolates identified as *N*. *flavescens*/*subflava* by 16S rRNA gene sequencing. The type strains of *N*. *sicca* and *N*. *mucosa* appeared closely related and were placed in a separate branch to the other strains.

The MLSA scheme was not used further and following personal communication (Dr. Julia Bennett, University of Oxford), analysis of the *rplF* gene was used as an alternative.

Table 17: Reference strains and genes for which MLSA PCR amplification and sequencing were possible. '+' sign indicates a successful result.

Reference type strain	***abcZ***	***adk***	***aroE***	***fumC***	***gdh***	***pdhC***	***pgm***
N. meningitidis	+	+	+	+	+	+	+
N. flavescens	+	+	+	+	+	+	+
N. subflava	+	+	+	+	+	+	+
N. sicca	+	+	+	+	+	+	+
N. elongata		+		+			
N. bacilliformis		+	+				
N. perflava	+	+	+	+	+	+	+
N. mucosa	+	+	+	+	+	+	+
N. weaveri		+					
N. polysaccharea	+	+	+	+	+	+	+

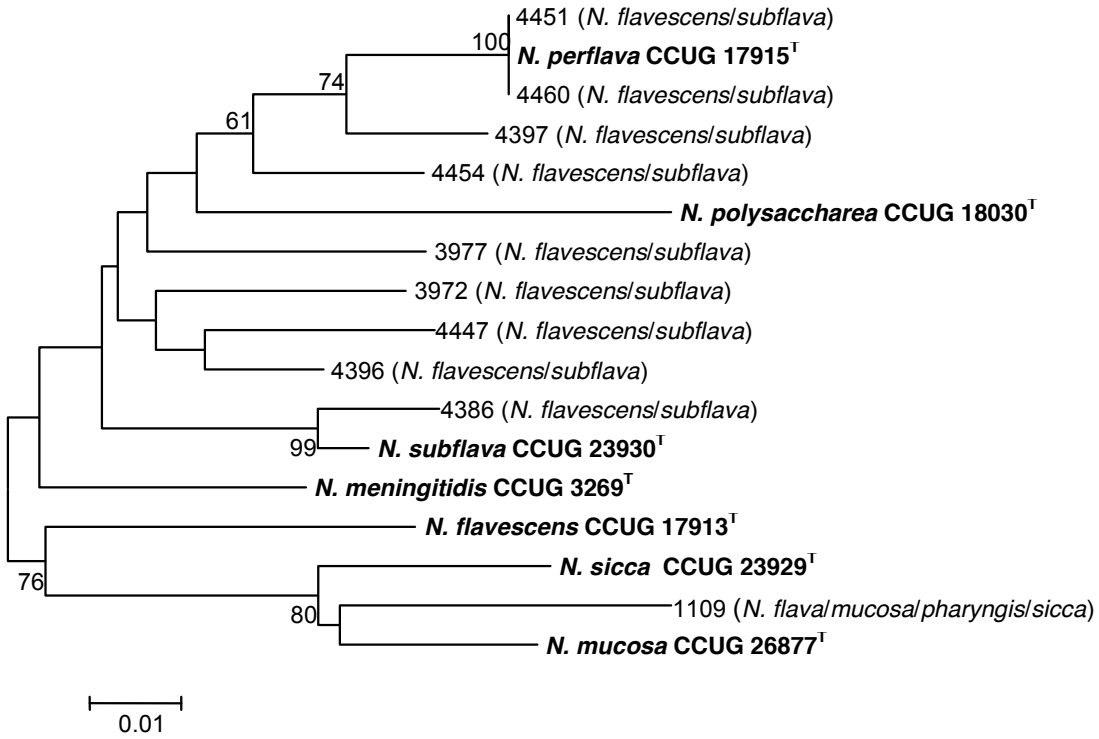

Figure 34: Phylogenetic tree based on *abcZ* gene sequences of *Neisseria* reference strains and oral isolates. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

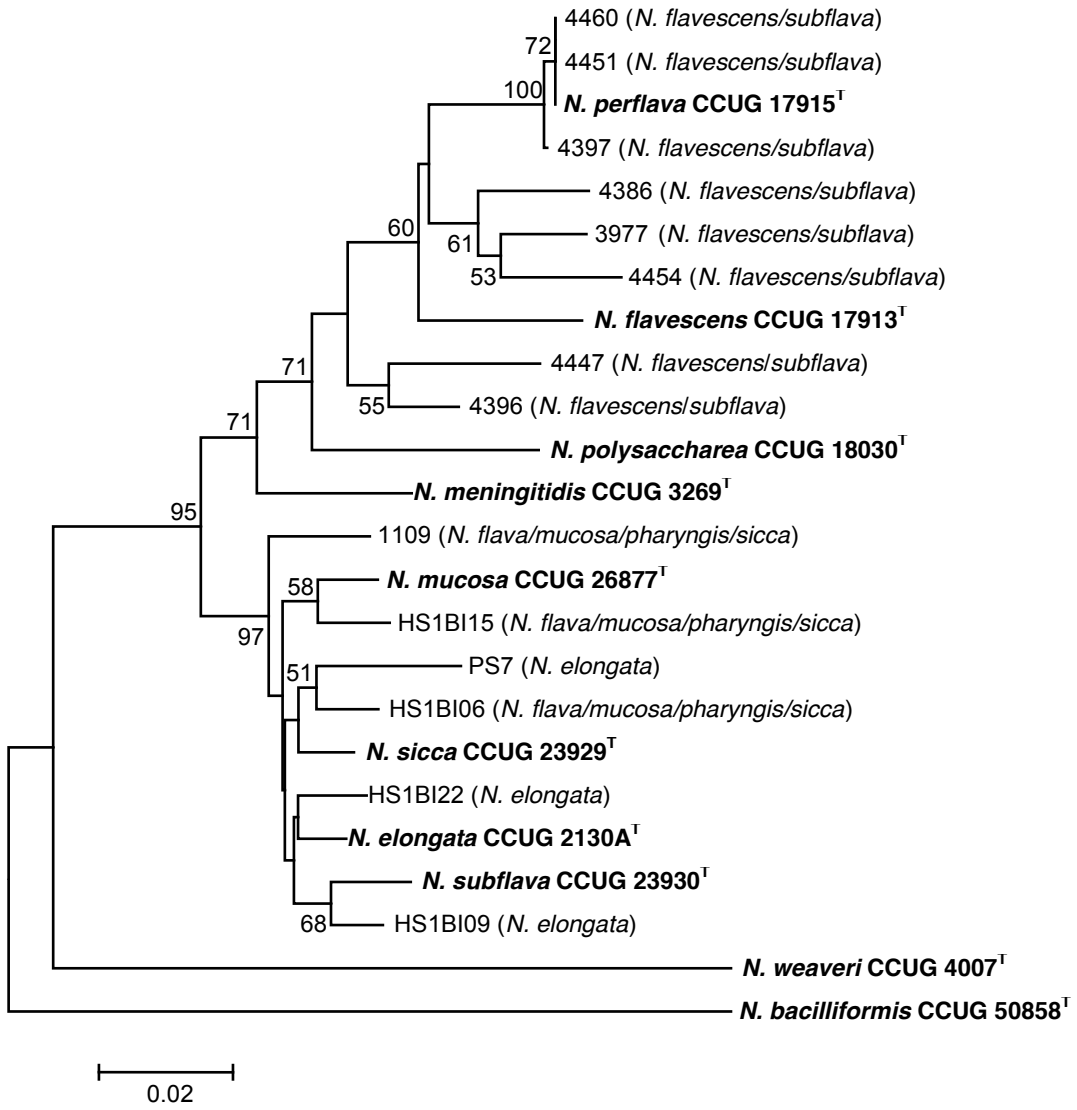

Figure 35: Phylogenetic tree based on *adk* gene sequences of *Neisseria* reference strains and oral isolates. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

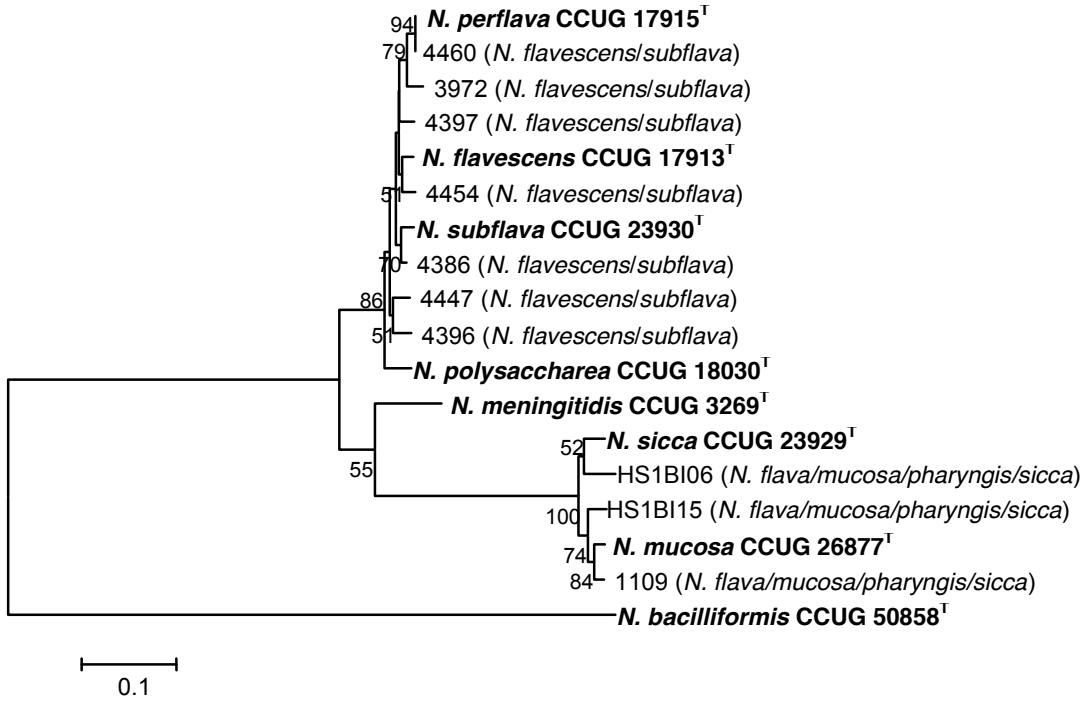

Figure 36: Phylogenetic tree based on *aroE* gene sequences of *Neisseria* reference strains and oral isolates. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

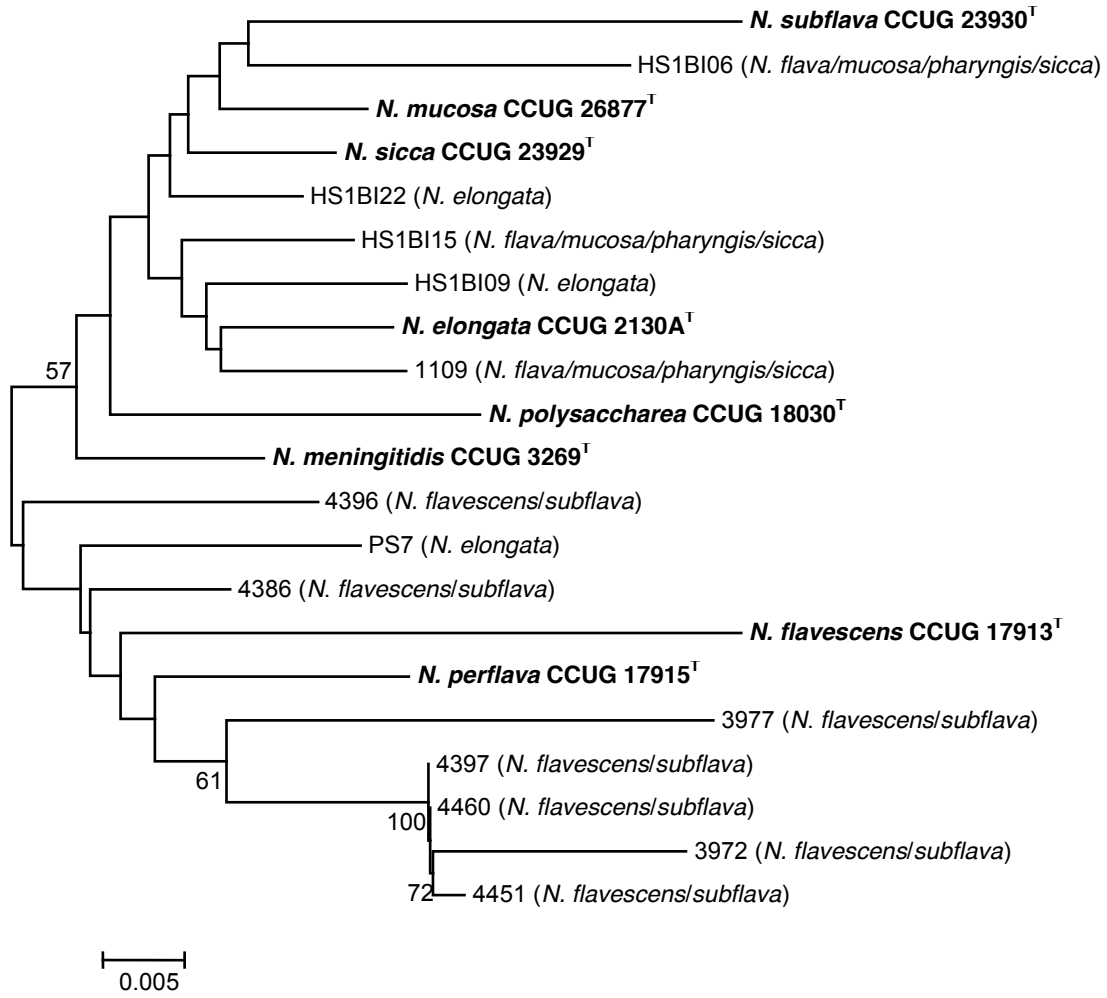

Figure 37: Phylogenetic tree based on *fumC* gene sequences of *Neisseria* reference strains and oral isolates. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

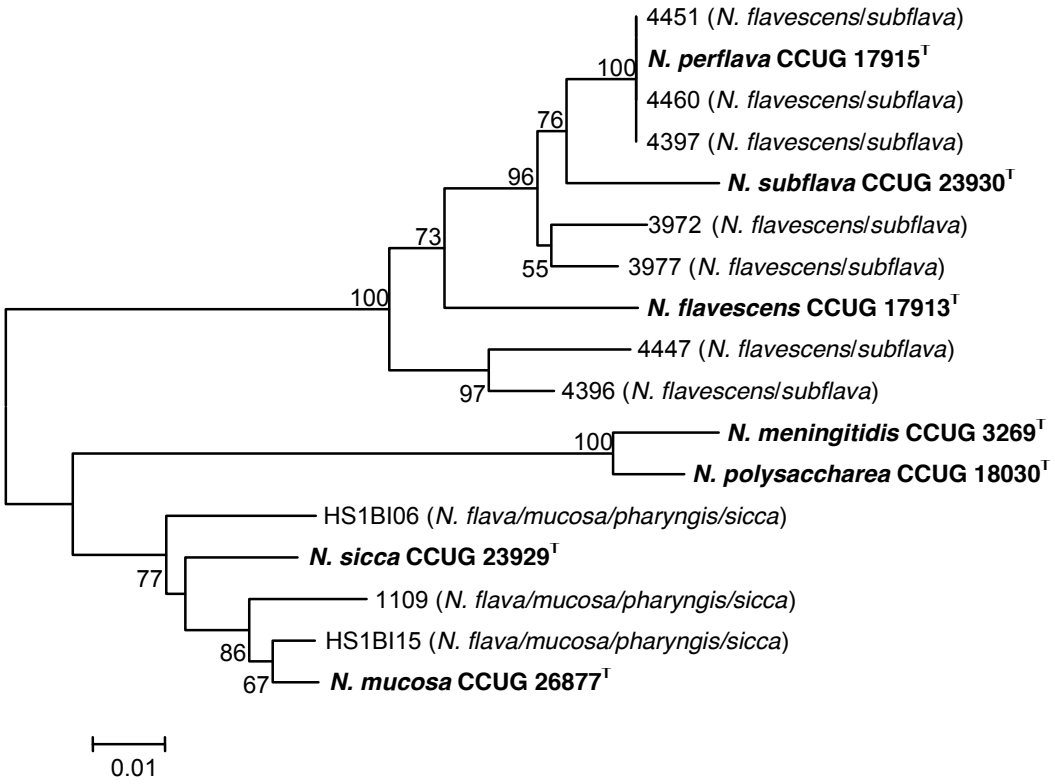

Figure 38: Phylogenetic tree based on *gdh* gene sequences of *Neisseria* reference strains and oral isolates. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

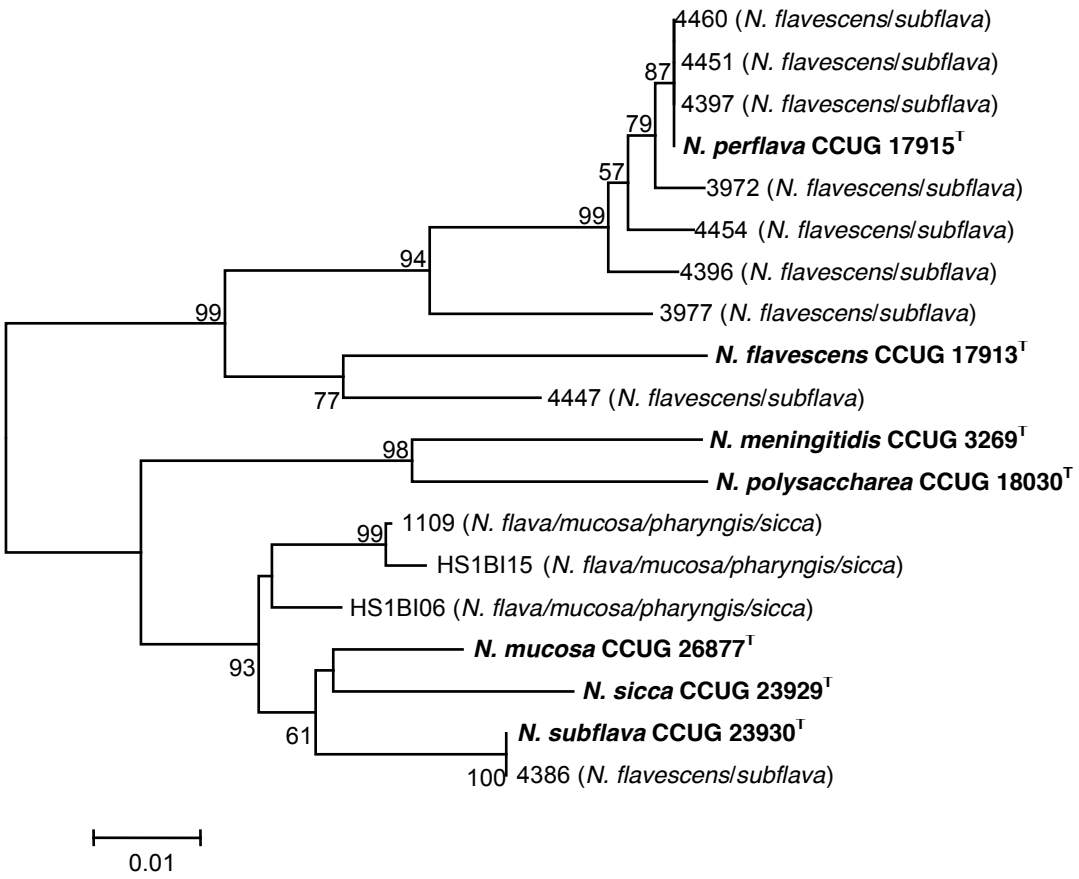

Figure 39: Phylogenetic tree based on *pdhC* gene sequences of *Neisseria* reference strains and oral isolates. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

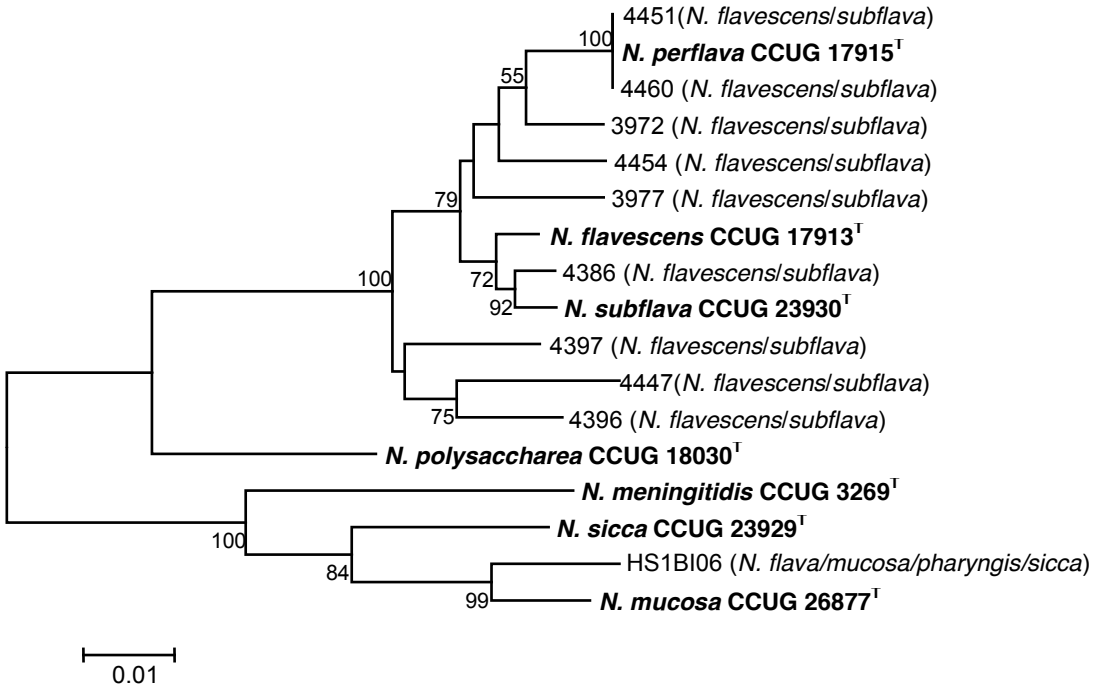

Figure 40: Phylogenetic tree based on *pgm* gene sequences of *Neisseria* reference strains and oral isolates. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

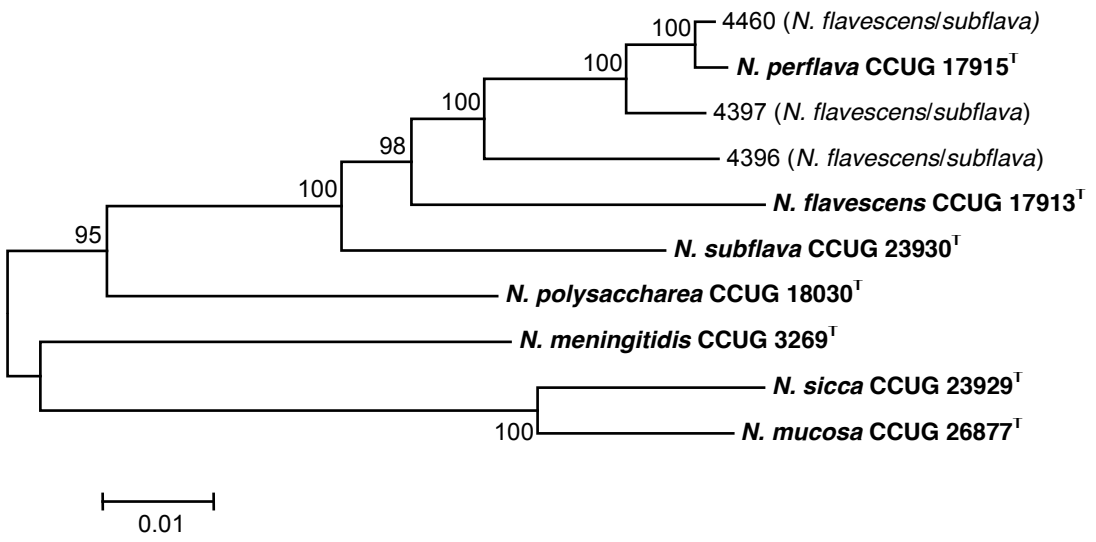

Figure 41: Phylogenetic tree based on concatenated sequences from 7 housekeeping genes of *Neisseria* reference strains and oral isolates. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

4.3.3 *Sequence analysis of the rplF gene*

Amplification and sequencing of a 413-bp fragment of the *rplF* gene using the original primers was successful for all of the reference strains except *N. bacilliformis* and *N. weaveri*. In addition, amplification was not possible for oral isolates identified as *Neisseria* sp. HOT018 and *Neisseria* sp. HOT020 by 16S rRNA gene sequencing. Modified primers enabled amplification and sequencing of the remaining isolates, with the exception of a single isolate identified as *Neisseria* sp. HOT018. Sections of a phylogenetic tree based on these sequences are shown in Figures 42-45. Visual inspection of the tree revealed some clustering that was congruent with the 16S rRNA tree, including three clusters that each contained only *N. elongata*, *N. bacilliformis,* or *Neisseria* sp. HOT020 isolates. The *N. elongata* and *N. bacilliformis* clusters also contained the reference type strains of those species. The *N. bacilliformis* and *Neisseria* sp. HOT020 clusters were highly divergent from all of the other *Neisseria* and emerged in a separate clade of the tree (Figure 42). Isolates that were identified as *N. flavescens*/*subflava* by 16S rRNA gene sequencing all fell into the same cluster in the *rplF* tree, along with the type strains of *N. flavescens*, *N. subflava*, and *N. perflava* (Figure 44). Isolates identified as *N. mucosa*/*sicca*/*pharyngis*/*flava* by 16S rRNA gene sequencing were separated into distinct groups in the *rplF* tree (Figure 43). The type strains of *N. mucosa* and *N. sicca* emerged in separate clusters and inspection of the sequence alignment revealed 16 different bases in the 413-base sequences. Isolates identified as *N. oralis*/HOT009/HOT015/HOT016 by 16S rRNA sequencing clustered into two groups in the *rplF* tree, each consisting of isolates recovered from a single different subject (Figure 43). One group appeared most closely related to *N. elongata,* whilst the other was most closely related to *N. sicca*.

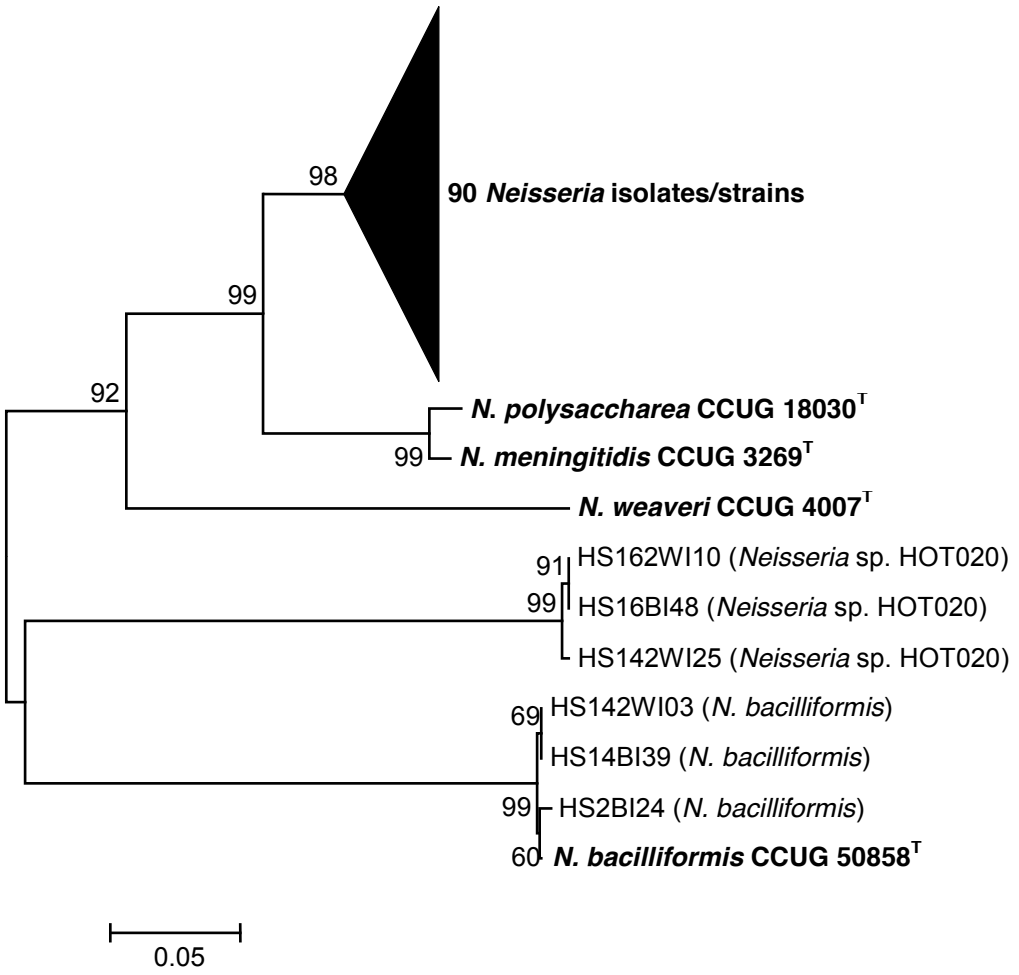

Figure 42: Section of a phylogenetic tree based on *rplF* gene sequences of *Neisseria* reference strains and oral isolates. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

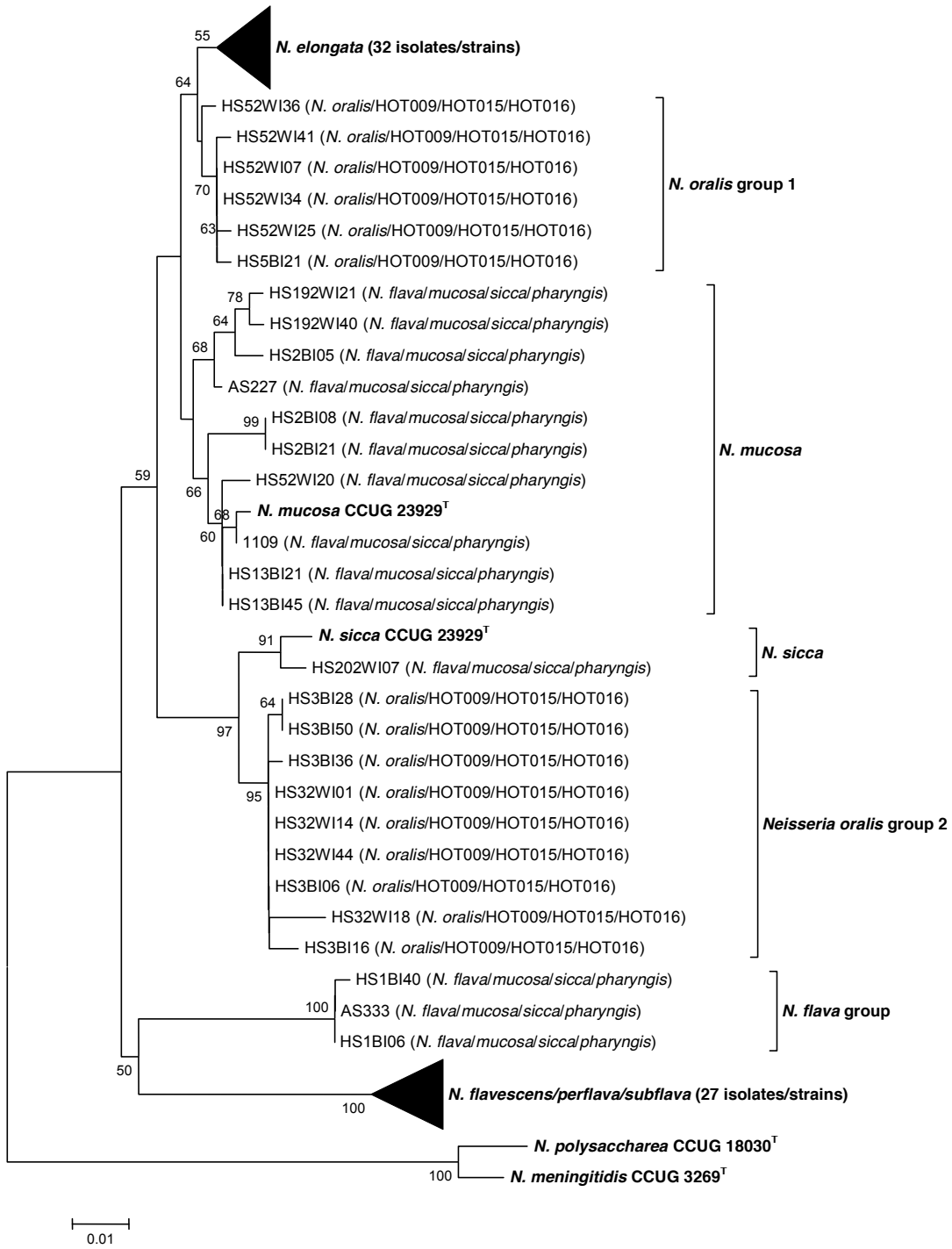

Figure 43: Section of a phylogenetic tree based on *rplF* gene sequences of *Neisseria* reference strains and oral isolates. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

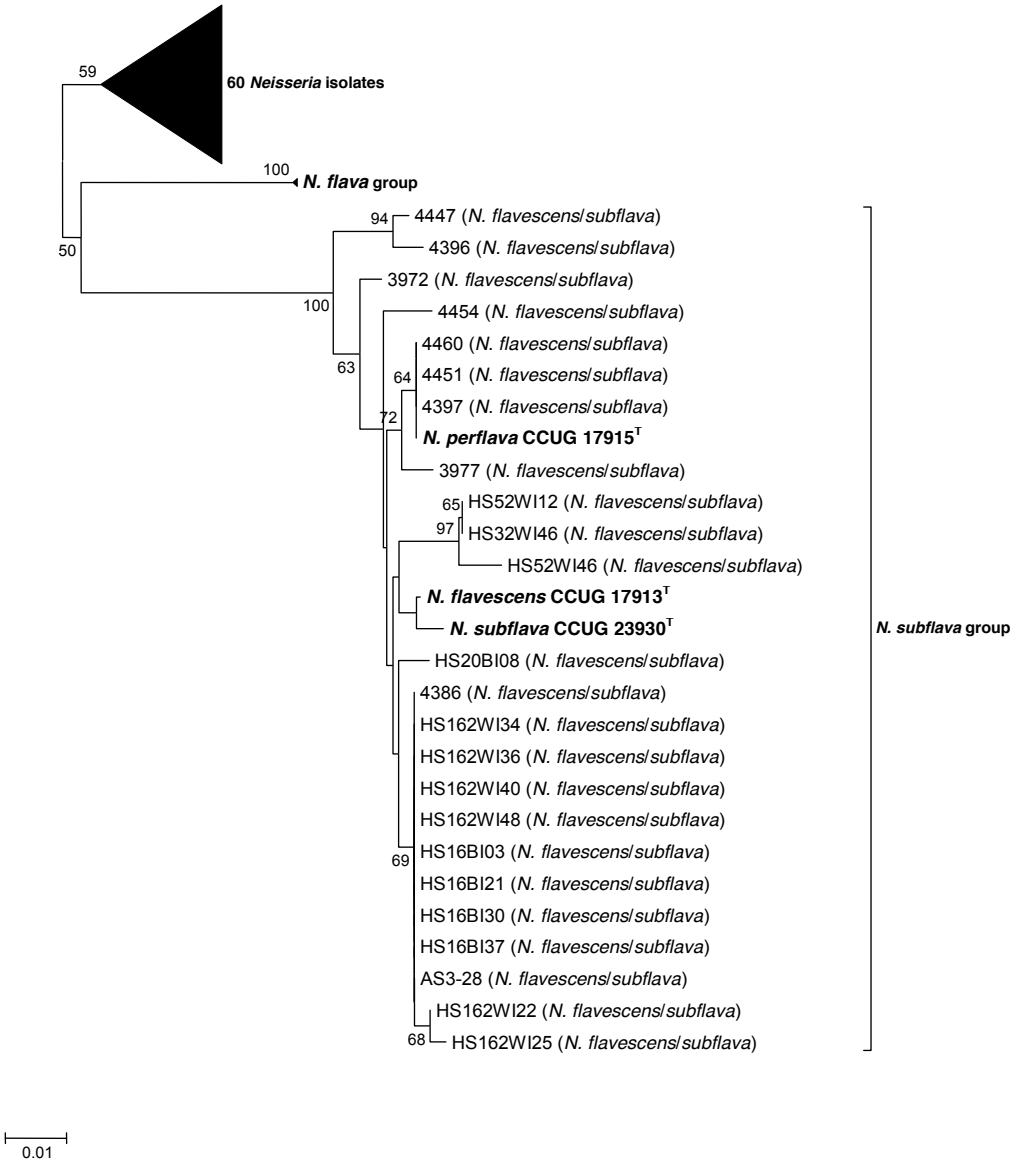

Figure 44: Section of a phylogenetic tree based on *rplF* gene sequences of *Neisseria* reference strains and oral isolates, showing the relationship of isolates in the *N. subflava* group. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

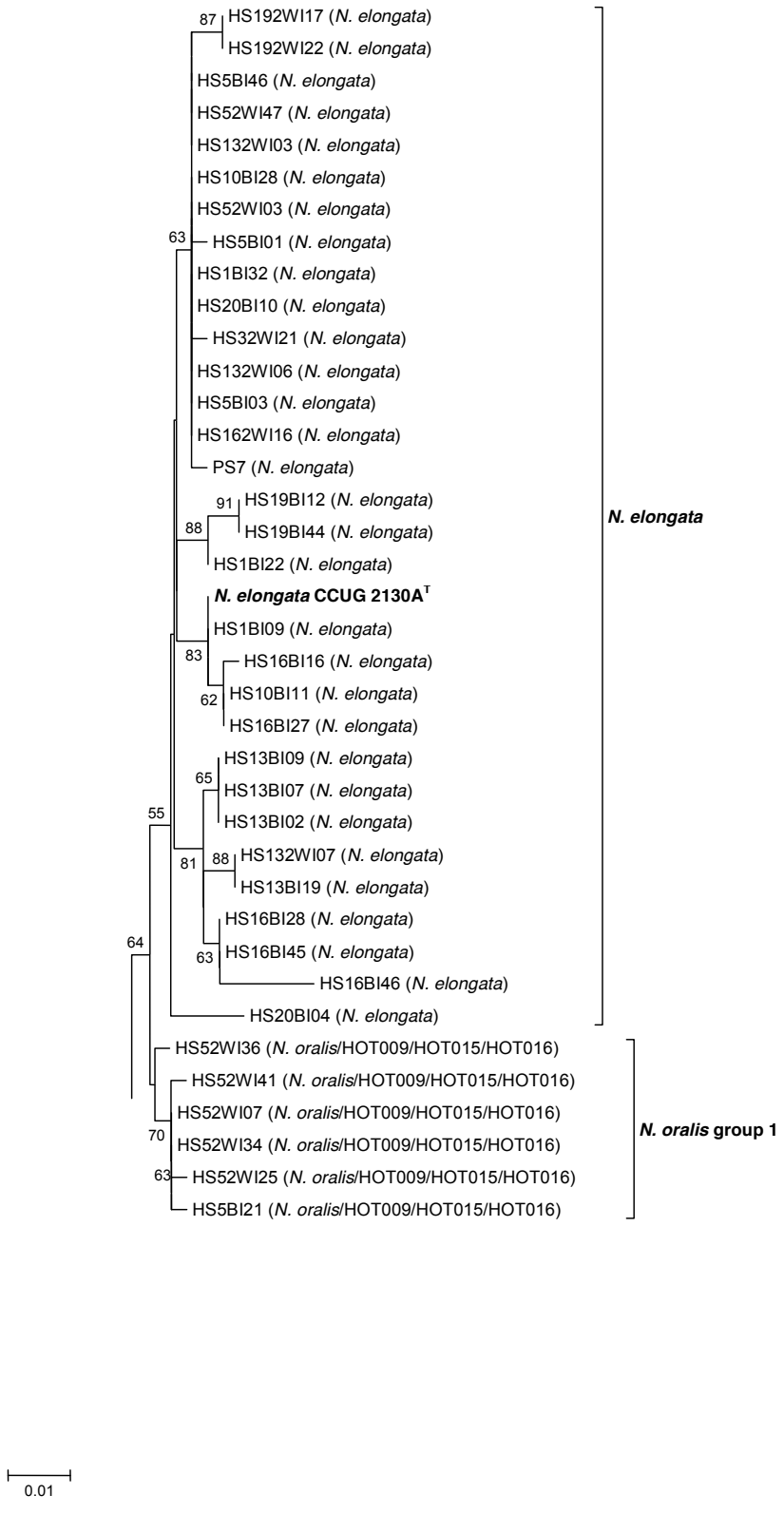

Figure 45: Section of a phylogenetic tree based on *rplF* gene sequences of *Neisseria* reference strains and oral isolates showing the relationship of isolates identified as *N. elongata* by 16S rRNA gene sequencing. Provisional 16S rRNA gene-based identities of isolates are shown in parentheses. The tree was constructed using the neighbour-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.

4.4 Discussion

This chapter evaluated alternative methods for the identification of oral *Neisseria* using a panel of reference strains and oral isolates. The ability to differentiate *Neisseria* species is important as the genus includes the pathogens *N. meningitidis* and *N. gonorrhoeae* as well as non-pathogenic commensal species. Reliable identification of different oral commensal species may also facilitate future work investigating their functions in oral microbial communities.

The MLSA scheme developed in this study, an adaptation of an MLST method used for *N. meningitidis* (Maiden et al., 1998), proved difficult to use with oral *Neisseria* isolates. There was extensive heterogeneity in the gene sequences, even at the more conserved sites used for primer design, resulting in the need for multiple variants of these primers for amplification and sequencing. The design of targeted primers was limited to strains for which genomic sequences were available and, even with the inclusion of degenerate nucleotides, these did not always amplify isolates that were assigned to the same species/group by 16S rRNA gene sequencing. It was not possible to design a set of primers for each gene that could be used for all of the different species/strains and this therefore limits the utility of the scheme. Previous studies attempting to expand MLST schemes from a single species to multiple species have reported similar difficulties (Miller et al., 2005, Rasback et al., 2007).

The individual loci used in the *Neisseria* MLSA scheme may be unreliable as discriminatory markers due to the lack of congruity seen in the single gene phylogenetic trees. It is possible that this incongruence was a result of horizontal gene transfer between different species. Indeed, inter-species recombinational exchanges within 'housekeeping' genes such as shikimate dehydrogenase (*aroE*) of

Neisseria species have been described previously (Zhou et al., 1997). The seven 'housekeeping' genes used here varied in their discriminatory ability with differing degrees of sequence divergence between isolates at individual loci, which may be explained by different rates of evolution and by recombinational events. Inter-species homologous recombination events occurring in a single 'housekeeping' locus (used for phylogenetic assignment) may also significantly alter the phylogenetic positioning of a strain. Hanage et al. (2005) demonstrated that concatenating such sequences can overcome the 'fuzzy' species boundaries brought about by recombination events. Owing to the difficulty in obtaining sequences for all seven genes for a number of the isolates in the present study, the concatenated tree was based on a very limited number of strains and was therefore of limited value.

Sequence analysis of the *rplF* gene was possible for all but one of the isolates attempted, and the majority of these could be amplified with the original primer set. This analysis was therefore readily applied to a large selection of different *Neisseria* strains and species and this is an advantage of this approach over the MLSA scheme. The clustering of isolates in the phylogenetic tree based on *rplF* sequences, largely agreed with that of the 16S rRNA gene, although some additional clusters within species/groups were resolved. The grouping together of all the isolates provisionally identified as *N. flavescens*/*subflava* along with the reference type strains of *N. flavescens*, *N. perflava* and *N. subflava* in the *rplF* tree is suggestive that these strains belong to the same species. This is also supported by the MLSA data, as in the majority of individual gene trees these isolates clustered together. Bennett et al. (2012) recently suggested that *N. flavescens* and *N. subflava* could be consolidated on the basis of similarities in 53 ribosomal protein genes. However, the authors did not include the type strain of *N. perflava* in their analyses and two of the reference

strains used that had been previously identified as *N*. *perflava* clustered with isolates identified as *N*. *mucosa*/*sicca*. Another *N*. *perflava* strain that was included, however, did cluster with *N*. *flavescens*/*subflava*. A phenotypic taxonomic study of *Neisseria* also indicated that *N*. *flavescens* and *N*. *subflava* may be the same species as these strains were grouped into the same 'phenon'(Barrett and Sneath, 1994). The *N*. *perflava* type strain however, was assigned to a separate phenon. If *N*. *flavescens*, *N*. *subflava* and *N*. *perflava* were to be consolidated then the name *N*. *subflava* would have precedence, as it was the first to be described. Isolates that were identified as *N*. *mucosa*/*sicca*/*flava*/*pharyngis* by 16S rRNA sequencing fell into distinct clusters in the *rplF* tree. The majority of these isolates grouped together with the *N*. *mucosa* reference type strain. Interestingly, three of these isolates had near identical *rplF* sequences and emerged on a well-resolved branch of the tree. As this branch did not contain a reference strain it is unclear which named species, if any, these may represent. The *N*. *mucosa* and *N*. *sicca* reference strains were resolved into separate clusters in the *rplF* tree, supporting their status as separate species despite sharing almost identical 16S rRNA gene sequences. However, a recent study using 53 ribosomal protein genes and 246 *Neisseria* core gene sequences found that these strains grouped together in the resulting concatenated trees and the authors suggested that these species should be consolidated (Bennett et al., 2013). The species *N*. *bacilliformis* and *Neisseria* sp. HOT020 were clearly distinct from all of the other *Neisseria* in the *rplF* tree. It would be of interest to sequence a greater number of isolates representative of these species, along with reference strains of other closely related genera, to determine if they warrant re-assignment to a distinct genus or genera.

Sequence analysis of the *rplF* gene has proved useful in investigating the phylogenetic relationships between oral *Neisseria* species. However, the use of a single locus is a limitation as horizontal gene transfer has the potential to distort these relationships. It would therefore be preferable to use multiple loci where possible. As DNA sequencing continues to fall in cost and becomes increasingly high-throughput, future studies could feasibly rapidly sequence the whole genomes of large numbers of isolates for phylogenetic analyses. This would overcome problems regarding the PCR amplification of multiple loci from different species encountered in this study. Instead, sequences from loci of interest can be extracted from the genomic sequences and used to create phylogenetic trees based on concatenated sequences, as has been demonstrated in rMLST (Jolley et al., 2012).

Chapter 5:
General discussion

Chapter 5: General discussion

The work described in this project comprehensively characterised the composition of the oral microbiota associated with periodontal health and determined the microbial changes associated with the initial stages of gingivitis. In addition, the potential for using health-associated oral species as probiotics for oral use was investigated, and this led to the identification of candidate probiotic strains.

The use of 454-pyrosequencing to monitor the microbiota in the experimental gingivitis study (Chapter 2) revealed the presence of a highly species-rich bacterial community in the plaque of healthy individuals. Between 201 and 383 species-level OTUs were detected in each plaque sample, representing a markedly higher richness and complexity than has been previously shown using conventional culture and Sanger sequencing methods (Ritz, 1967, Aas et al., 2005, Bik et al., 2010). Whilst early pyrosequencing studies detected in the order of thousands of species-level OTUs (Keijser et al., 2008) in individual plaque and saliva samples, the implementation of de-noising strategies to reduce sequencing error in recent pyrosequencing studies has led to richness estimates more similar to that of the present study (Griffen et al., 2012, Abusleme et al., 2013). The tremendous richness and complexity of plaque communities found in this study highlights the limitation of solely using traditional closed-ended approaches to study the oral microbiome. This includes methods such as culture using selective media, targeted PCR, and checkerboard DNA-DNA hybridisation, all of which pre-select for particular taxa.

A detailed analysis of the plaque communities was achieved in this work with identification of sequences to the species-level for the most part. This analysis confirmed the high prevalence and relative abundance of a number of oral taxa

commonly isolated from plaque in culture, including the early colonising species *S. sanguinis*, and *A. naeslundii* (Moore et al., 1982). However, the data also showed that other species such as *C. matruchotii* and *L. buccalis* are both prevalent and abundant members of plaque communities, and thus warrant greater attention. In addition, the analyses detected novel phylotypes present in multiple individuals in health and disease that, although present at a low abundance, may be of importance. Recent work in mice has demonstrated that *P. gingivalis* can, when present at low abundance, induce a shift in the microbiota from a benign to dysbiotic state (Hajishengallis et al., 2011). Relatively minor constituents of the microbial community may therefore play an important role in disease initiation and/or progression. Together, the results of the current project have furthered knowledge of which species are present in plaque, both in periodontal health and disease.

An interesting finding of this study was the considerable inter-individual variability in bacterial community membership and the relatively small shared, or 'core', microbiota that appeared to be present at the species-level. Only eight species-level OTUs were detected in all of the healthy individuals, indicating the likelihood of functional redundancy among different species in the microbiota. This finding is corroborated by data from the Human Microbiome Project Consortium (2012) (HMP) that has highlighted the uniqueness of every individual's microbiome. In those studies, analysis of 4788 specimens from 242 healthy individuals did not reveal any taxa that were present in all of the individuals. In addition, within subject variation of community structure over time was shown to be smaller than variation between subjects. Interestingly, it was further shown that the individuals' microbiomes differed not only in terms of the membership and abundance of species, but that the same species (for example the oral species *S. mitis*) in different

individuals showed marked strain-level genomic variation. The variability between different individuals' plaque communities warrants further exploration in a larger population. It would be interesting, for instance, to determine, if healthy individuals can be clustered into groups according to the membership and structure of their oral microbiome, in a similar manner to the 'enterotypes' that have been described for the human gut microbiome (Arumugam et al., 2011). A recent study, however, using data generated from the HMP and the Metagenomics of the Human Intestinal Tract (MetaHIT) consortia, found that samples fell into gradients based on the abundance of common taxa rather than the well-defined enterotype clusters reported previously (Koren et al., 2013). The authors found that the methodology used for analysis, such as the PCoA distance metric and OTU picking algorithms, affected the results, with some methods supporting enterotype clustering more strongly than others. Recent data based on terminal restriction fragment length polymorphism (t-RFLP) and pyrosequencing analysis of saliva and plaque samples has suggested that the oral microbiome differs according to ethnicity (Mason et al., 2013). However, it is difficult to determine if this variability can be attributed to genetic differences or differences in a wide range of possible environmental factors, including dietary intake of fermentable carbohydrates and age. The authors gave no indication that the groups had been age or gender-matched, although they excluded individuals under 18 years of age, and did not provide data to support the assertion that all of the subjects were periodontally healthy. Although the maximum threshold for gingival inflammation was stated, differences in gingival inflammation between the groups could have, therefore, been responsible for the microbial differences found. The consortia of species proposed as characteristic of the ethnic groups achieved the best

prediction likelihood of 65% for African Americans, but dropped to 47% for "Latinos", 45% for Caucasians and only 33% for Chinese.

The high degree of inter-individual variability in the composition of the oral microbiota in health found in this study, and in the HMP cohort, suggests that a personalised approach to the prevention and treatment of periodontal disease may be useful. For example, the oral microbiota of an individual could be screened for the presence of specific putative periodontal pathogens using deep sequencing methods. Should significant numbers / proportions of such species be detected, intervention in the form of probiotics, or targeted antimicrobial therapy, could be considered in order to manipulate the composition of the microbiota. Probiotic strains with demonstrated antagonism against periodontitis-associated species may be particularly useful in such cases. In addition, if groupings of individuals based on the composition of their oral microbiome, analogous to the gut 'enterotypes', are revealed, this could potentially help to inform the most appropriate form of prevention or therapy for a given individual.

The pyrosequencing data showed that, following the withdrawal of oral hygiene there was a shift in community structure and a significant increase in the diversity of the plaque bacteria, as gingivitis developed. This supports the findings of previous experimental gingivitis studies (Loe et al., 1965, Moore et al., 1982) based on microscopy and culture that reported changes in the predominant taxa present during the transition from health to gingivitis. There have been no other experimental gingivitis studies using molecular methods with which comparison is possible. One pyrosequencing study, however, reported significant differences in the microbial composition of plaque of three healthy subjects compared to that of three subjects with naturally occurring gingivitis (Huang et al., 2011). A number of new gingivitis-

associated OTUs were identified in the present study, included the unnamed species *Lachnospiraceae* sp. [G-2] HOT100 and *Lautropia* sp. HOTA94. The association of an OTU, identified as *F. nucleatum,* with gingivitis confirms previous work using culture that reported this species to increase significantly in individuals, and sites, with gingival inflammation (Moore and Moore, 1994). The organism most strongly associated with health, based on the experimental gingivitis data, was the Gram-positive facultative anaerobe *Rothia dentocariosa,* of the phylum *Actinobacteria*. This confirms the findings of recent studies that have associated this species with periodontal health (Griffen et al., 2012, Abusleme et al., 2013). *R. dentocariosa* reportedly grows more rapidly in aerobic conditions than in anaerobic conditions (Von Graevenitz, 2004), and this could explain its high relative abundance at baseline when the plaque biofilms were relatively thin and oxygen concentrations would likely have been higher than in one- or two-week biofilms. Previous work has shown that the redox potential of plaque falls as it develops over time (Kenney and Ash, 1969). It would be interesting to determine if, following the reinstatement of oral hygiene and reversal of experimental gingivitis, the microbiota is restored to its original state. Future experimental gingivitis studies could continue to monitor the microbiota as gingivitis is reversed to address this. In addition, follow-up work might aim to examine the composition of the microbiota in individuals with mild naturally occurring gingivitis and monitor the microbial changes when their gingivae are restored to health by effective oral hygiene.

This study was consistent with previous studies reporting significant differences in the community membership and structure of plaque from chronic periodontitis patients compared to healthy subjects (Socransky et al., 1998, Kumar et al., 2006, Griffen et al., 2012). Determination of differentially abundant OTUs

between healthy subjects and chronic periodontitis patients resulted in the identification of 22 periodontitis-associated OTUs. These OTUs included many previously associated putative periodontal pathogens, including *P. gingivalis*, *F. alocis* and *T. forsythia*, although the association of some OTUs such as the uncultured phylotype *Leptotrichia* sp. HOT B57 were new findings. The genus *Leptotrichia* comprises a group of Gram-negative, filamentous, saccharolytic, predominantly anaerobic bacteria (some strains are able to grow aerobically with $C0_2$) (Eribe and Olsen, 2008). Many members of the genus are currently uncultured and thus only represented by 16S rRNA gene sequences, as in the case of *Leptotrichia* sp. HOT B57. *Leptotrichia* spp. have previously been associated with naturally-occurring gingivitis (Huang et al., 2011) and comprised up to four percent of the cultivable microbiota in the subgingival plaque samples of subjects with refractory periodontitis (Colombo et al., 1998). Interestingly, *Leptotrichia* sp. HOT B57 (*Leptotrichia* sp. oral clone EX103) was previously detected in the subgingival plaque of subjects with necrotizing ulcerative periodontitis, but not in that of subjects without the infection, in a 16S rRNA gene cloning and sequencing study (Paster et al., 2002).

Whilst this study successfully used 454-pyrosequencing to provide a detailed insight into the composition of the oral microbiota in periodontal health and disease, there are a number of limitations associated with the methodology that should be taken into account. The analysis of bacterial communities using 16S rRNA gene sequencing can be subject to biases introduced at different stages of library preparation. For example, DNA extraction can be biased against certain taxa, particularly Gram-positive cells that are resistant to cell lysis (Yuan et al., 2012). PCR amplification may also introduce biases by the use of broad-range primers that

have mismatches in the primer binding sequence in some species (Sim et al., 2012), or due to the high G+C content in the 16S rRNA genes of certain taxa (de Lillo et al., 2006). Although it is difficult to completely overcome these biases, a useful means of monitoring them is through the analysis of mock bacterial communities where the composition of the community is known prior to sequencing (Diaz et al., 2012). Another advantage of sequencing a mock community is that sequencing errors that can lead to an over-estimation of the species/OTU richness may also be assessed. It would therefore be useful if future studies were to include a mock community sample along with every pyrosequencing run, so that steps may then be taken to attempt to reduce any observed biases.

Another limitation associated with 16S rRNA gene sequencing studies relates to the difficulty in defining bacterial 'species' (Rossello-Mora and Amann, 2001), particularly following the recognition that many bacteria engage in horizontal gene transfer (Ochman et al., 2000). As noted in Chapter 2, there is no consensus sequence identity threshold to define a species based on the 16S rRNA gene. As a result, different studies often use different percentage identity thresholds to cluster sequences into OTUs that approximate species. In addition, some species cannot be differentiated by their 16S rRNA gene sequences, as is the case with the majority of oral *Neisseria* species (Chapter 4), and this is a further challenge. Strains or sub-species of a given species may differ markedly in terms of their functional potential. For example, some strains of a particular species may be capable of producing antimicrobial compounds such as bacteriocins or carry genes conferring antibiotic resistance. Similarly, a single clone of a particular species can be significantly more virulent than the remainder of the population. A good example of this is the JP2 clone of *A. actinomycetemcomitans*, which is highly leukotoxic and is associated

with aggressive periodontitis in susceptible populations (Haubek, 2010). Cataloguing the bacterial species present in the oral microbiome is not, therefore, sufficient to fully understand its role in periodontal health and disease.

It cannot be determined from the results of this study if the OTUs found to be associated with gingivitis and chronic periodontitis play a causal role, or increase in disease as a result of the distinct environment that emerges following gingival inflammation and destruction of periodontal tissue. As discussed in Chapter 1, considerable efforts have been made to study the pathogenicity of putative periodontal pathogens, in particular the 'red complex' species: *P. gingivalis*, *T. forsythia* and *T. denticola*, to better understand their association with disease. To this end, many studies have focused on the identification of virulence factors, such as tissue-damaging proteases, expressed by these species. Some studies have used animal models to investigate their role in periodontal disease. For example, work using a murine model has shown that *P. gingivalis* can induce periodontal bone loss in specific pathogen-free mice, but not in germ-free mice, indicating that members of the commensal mouse oral microbiota are essential for destructive disease (Hajishengallis et al., 2011). In addition, it was shown that complement was essential for periodontal bone loss, as mice deficient in the anaphylatoxin (polypeptides generated during complement activation) receptors C3aR and C5aR were unaffected. On the basis of these findings, it was proposed that *P. gingivalis* can modulate the host complement system, thereby triggering a change in the amount and composition of the normal commensal microbiota, such that it becomes dysbiotic, or disease-associated (Darveau et al., 2012). It is yet to be shown if other putative periodontal pathogens can function as so-called 'community activists' and induce destructive disease in this manner. Interestingly though, recent work has revealed that NI1060,

an un-named commensal species-level taxon of the *Pasteurellaceae*, can activate a pattern recognition receptor leading to periodontal bone loss in mice. Jiao et al. (2013) used a murine model that included a silk ligature placed around the molar teeth of the mice to encourage the accumulation of bacteria. After ten days, periodontal bone loss was observed. By using knockout mice and comparing them to wild-type mice, the authors showed that periodontal bone loss required the pattern recognition receptor Nod1 (nucleotide binding oligomerization domain-containing protein 1). NI1060 dominated the bacterial communities at ligature sites and was able to stimulate Nod1. Furthermore, unlike *P. gingivalis*, NI1060 was able to induce periodontal bone loss in germ-free mice. Intriguingly, genome sequencing of this organism revealed that it is related to *A. actinomycetemcomitans*, with which it shares a total of 1536 genes. A clone of *A. actinomycetemcomitans* (JP2) has been strongly associated with aggressive periodontitis in some populations (Haubek, 2010).

This project also investigated the potential for using isolates of oral bacteria from healthy subjects as probiotics for the prevention and control of periodontal disease. Strains of the health-associated oral species *S. cristatus* inhibited the growth of putative periodontal pathogens in the deferred antagonism assay described in Chapter 3 and thus have potential use as oral probiotics. This is the first report of bacteriocin-like inhibitory substance activity (BLIS) detected in this species, although activity has been previously reported in other oral viridans streptococci including *S. salivarius* and *S. mutans* (Dempster and Tagg, 1982, Tagg, 2004). As discussed in Chapter 1, there is currently limited evidence supporting the efficacy of probiotics for the prevention and control of dental disease. This may, in part, be because the majority of probiotic strains tested in clinical studies were originally for

use in the distal gut and are not commonly found in the oral cavity. It is possible then, that these strains do not colonise and persist on oral surfaces and so any beneficial effect conferred may be very limited. An exception to this is the probiotic strain K12 of the species *S. salivarius*, which is commonly found on mucosal oral surfaces and in the saliva of healthy subjects (Kazor et al., 2003, Aas et al., 2005). This strain was shown to inhibit the growth of halitosis-associated oral bacteria *in vitro* and to reduce parameters of halitosis (volatile sulphur compounds) in humans (Burton et al., 2006). The *S. cristatus* strains that showed inhibitory activity in this study were isolated from the plaque of healthy individuals, and the pyrosequencing data showed that this species was a common member of the microbiota. Consequently, the strains identified in this project may be better candidates for testing as potential periodontal probiotics in clinical trials than those that have been tested with limited result, to date. However, further characterisation, including a safety assessment, of these strains is first necessary.

Continued improvements in high-throughput sequencing technology are allowing higher numbers of sequences to be obtained at a lower cost. As a result it is becoming increasingly practicable to apply metagenomics and metatransciptomics to the study of complex microbial communities. In contrast to 16S rRNA gene sequencing studies, these techniques can reveal valuable information regarding the function of microbial communities. Metagenomics involves the analysis of the whole genomic content of a microbial community by direct 'shotgun' sequencing. The sequence reads and contigs obtained can be functionally annotated using, for example, the Kyoto Encyclopedia for Genes and Genomes (KEGG) and metabolic pathways can be reconstructed from the data (Abubucker et al., 2012). Metagenomic data from faecal samples of 124 individuals in the HMP has resulted in the

identification of a set of 3.3 million non-redundant genes that are likely to be important for normal functioning of the gut microbiota (Qin et al., 2010). An interesting finding of that study was that individuals who had inflammatory bowel disease (IBD) had 25% fewer genes than healthy individuals, indicating a potential dysfunction of the microbiome in IBD. Recent studies have begun to use metagenomics to investigate the oral microbiota in health and chronic periodontitis (Liu et al., 2012, Wang et al., 2013). Although these studies were based on a small number of subjects they provided an interesting insight into the potential functional differences between health- and periodontitis-associated plaque communities. Wang et al. (2013), for example, found that genes involved in the synthesis of lipopolysaccharides, bacterial chemotaxis and flagellar assembly were significantly more abundant in the periodontitis-associated communities. A weakness of metagenomic studies is that they do not provide information concerning gene expression and so it cannot be inferred which genes or species are active in the community, only their presence and abundance. Knowledge of which genes are actively expressed by which taxa, may improve understanding of the mechanisms that lead to disease.

Metatranscriptomics, which involves the sequencing of the mRNA content of a sample, provides a method for profiling community gene expression. This is a potentially valuable technique as the means by which communities respond to changes in the environment can be monitored. If applied to the study of periodontal disease, metatransciptomics could be used to monitor changes in gene expression of plaque biofilms accompanying the transition from health to gingivitis i.e. in experimental gingivitis. There have been no reported metatranscriptomic studies of the human oral microbiome, to date. However, one study used metatranscriptomics

to elucidate changes in gene expression of a biofilm composed of five oral species, following the introduction of *P. gingivalis* and *A. actinomycetemcomitans*. (Frias-Lopez and Duran-Pinedo, 2012). The authors found that these putative pathogens altered the gene expression profile of the 'healthy' biofilm with an up-regulation of genes encoding molecular chaperones that were thought to be indicative of increased stress, as well as ABC-transporters and transposases.

Other approaches that have the potential to provide insights into the function of oral bacterial communities include metaproteomics and metabolomics. Improvements in peptide separation and mass spectrometry have recently enabled metaproteomic studies of the human microbiome to be performed. One such study investigated the meta-proteome of the salivary microbiota (Rudney et al., 2010). Rudney et al. (2010) found that the majority of the peptides were assigned to five bacterial phyla (*Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Spirochaetes*) and 17 % of the peptides were assigned to the genus *Streptococcus*. Interestingly, the authors reported that approximately six percent of the peptides were from 'exotic', or exogenous, taxa, including the aphid endosymbiont *Buchnera aphidicola*. Analysis of the peptides at a functional level revealed that most were involved in translation, glycolysis, amino acid metabolism, and energy production. Metabolomics involves the identification and quantification of the metabolites present in a sample, typically using capillary electrophoresis and mass spectrometry. This approach has been used, for example, to compare the metabolites present in gingival crevicular fluid from healthy, gingivitis and chronic periodontitis sites (Barnes et al., 2009). The authors reported that inosine, hypoxanthine, xanthine, guanosine, and guanine were increased at diseased sites indicating an up-regulation of the purine degradation pathway and an increase in associated reactive oxygen

species; which have a suggested role in periodontal tissue destruction (Waddington et al., 2000).

Whilst techniques such as metagenomics and metatranscriptomics are undoubtedly powerful, there are a number of associated challenges that future work will need to overcome. Perhaps the most significant challenge is the requirement for reference genomes of the large number of as-yet uncultured oral taxa in order to enable comprehensive analysis of the datasets. Attempting to grow the taxa that are currently refractory to culture, and sequencing their genomes, is therefore an important objective (Vartoukian et al., 2010). Due to the genomic variation found within species discussed earlier, there is also a need to obtain multiple genomes for each species. Another increasingly viable approach to obtain reference genomes is single-cell genomics, which bypasses the need for culture (Rinke et al., 2013). A further limitation associated with "meta-omics" approaches is the computational time and storage required for the enormous volumes of data generated. Despite these challenges, the combined use of metabolomics, metagenomics, metaproteomics and metatranscriptomics in future investigations of the oral microbiome has the potential to enhance our understanding of the mechanisms underlying the initiation and progression of periodontal disease.

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Appendix A: 454 pyrosequencing primers

Primer name	Golay barcode	Full primer 5'-3'
27FYM-A-1	AACTCGTCGATG	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACTCGTCGATGAGAGTTTGATYMTGGCTCAG
27FYM-A-2	AACTGTGCGTAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACTGTGCGTACAGAGTTTGATYMTGGCTCAG
27FYM-A-3	AAGAGATGTCGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGATGTCGAAGAGTTTGATYMTGGCTCAG
27FYM-A-4	AAGCTGCAGTCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGCTGCAGTCGAGAGTTTGATYMTGGCTCAG
27FYM-A-5	AATCAGTCTCGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGAATCAGTCTCGTAGAGTTTGATYMTGGCTCAG
27FYM-A-6	AATCGTGACTCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGAATCGTGACTCGAGAGTTTGATYMTGGCTCAG
27FYM-A-7	ACACACTATGGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACACTATGGCAGAGTTTGATYMTGGCTCAG
27FYM-A-8	ACACATGTCTAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACATGTCTACAGAGTTTGATYMTGGCTCAG
27FYM-A-9	ACACGAGCCACA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACGAGCCACAAGAGTTTGATYMTGGCTCAG
27FYM-A-10	ACACGGTGTCTA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACGGTGTCTAAGAGTTTGATYMTGGCTCAG
27FYM-A-11	ACACTAGATCCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACTAGATCCGAGAGTTTGATYMTGGCTCAG
27FYM-A-12	ACACTGTTCATG	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACTGTTCATGAGAGTTTGATYMTGGCTCAG
27FYM-A-13	ACAGACCACTCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGACCACTCAAGAGTTTGATYMTGGCTCAG
27FYM-A-14	ACAGAGTCGGCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGAGTCGGCTAGAGTTTGATYMTGGCTCAG
27FYM-A-15	ACAGCAGTGGTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGCAGTGGTCAGAGTTTGATYMTGGCTCAG
27FYM-A-16	ACAGCTAGCTTG	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGCTAGCTTGAGAGTTTGATYMTGGCTCAG
27FYM-A-17	ACAGTGCTTCAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGTGCTTCATAGAGTTTGATYMTGGCTCAG

Appendix A

Primer name	Golay barcode	Full primer 5'-3'
27FYM-A-18	ACAGTTGCGCGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACAGTTGCGCGAAGAGTTTGATYMTGGCTCAG
27FYM-A-19	ACATCACTTAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATCACTTAGCAGAGTTTGATYMTGGCTCAG
27FYM-A-20	ACATGATCGTTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATGATCGTTCAGAGTTTGATYMTGGCTCAG
27FYM-A-21	ACATGTCACGTG	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATGTCACGTGAGAGTTTGATYMTGGCTCAG
27FYM-A-22	ACATTCAGCGCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACATTCAGCGCAAGAGTTTGATYMTGGCTCAG
27FYM-A-23	ACCACATACATC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACCACATACATCAGAGTTTGATYMTGGCTCAG
27FYM-A-24	ACCAGACGATGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACCAGACGATGCAGAGTTTGATYMTGGCTCAG
27FYM-A-25	CAACACGCACGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAACACGCACGAAGAGTTTGATYMTGGCTCAG
27FYM-A-26	CAACTATCAGCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAACTATCAGCTAGAGTTTGATYMTGGCTCAG
27FYM-A-27	CAACTCATCGTA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAACTCATCGTAAGAGTTTGATYMTGGCTCAG
27FYM-A-28	CAAGATCGACTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAAGATCGACTCAGAGTTTGATYMTGGCTCAG
27FYM-A-29	CACACGTGAGCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACACGTGAGCAAGAGTTTGATYMTGGCTCAG
27FYM-A-30	CACAGCTCGAAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACAGCTCGAATAGAGTTTGATYMTGGCTCAG
27FYM-A-31	CACAGTGGACGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACAGTGGACGTAGAGTTTGATYMTGGCTCAG
27FYM-A-32	CACATCTAACAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACATCTAACACAGAGTTTGATYMTGGCTCAG
27FYM-A-33	CACATTGTGAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACATTGTGAGCAGAGTTTGATYMTGGCTCAG
27FYM-A-34	CACGACAGGCTA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGACAGGCTAAGAGTTTGATYMTGGCTCAG
27FYM-A-35	CACGGACTATAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGGACTATACAGAGTTTGATYMTGGCTCAG
27FYM-A-36	CACGTCGATGGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGTCGATGGAAGAGTTTGATYMTGGCTCAG

Appendix A

Primer name	Golay barcode	Full primer 5'-3'
27FYM-A-37	CACGTGACATGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACGTGACATGTAGAGTTTGATYMTGGCTCAG
27FYM-A-38	CACTACTGTTGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACTACTGTTGAAGAGTTTGATYMTGGCTCAG
27FYM-A-39	CACTGGTATATC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACTGGTATATCAGAGTTTGATYMTGGCTCAG
27FYM-A-40	CACTGTAGGACG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCACTGTAGGACGAGAGTTTGATYMTGGCTCAG
27FYM-A-41	CAGACATTGCGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGACATTGCGTAGAGTTTGATYMTGGCTCAG
27FYM-A-42	CAGACTCGCAGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGACTCGCAGAAGAGTTTGATYMTGGCTCAG
27FYM-A-43	CAGAGGAGCTCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAGGAGCTCTAGAGTTTGATYMTGGCTCAG
27FYM-A-44	CAGATACACTTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATACACTTCAGAGTTTGATYMTGGCTCAG
27FYM-A-45	CAGATCGGATCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATCGGATCGAGAGTTTGATYMTGGCTCAG
27FYM-A-46	CAGCACTAAGCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGCACTAAGCGAGAGTTTGATYMTGGCTCAG
27FYM-A-47	ACTCGATTCGAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTCGATTCGATAGAGTTTGATYMTGGCTCAG
27FYM-A-48	ACTCGCACAGGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTCGCACAGGAAGAGTTTGATYMTGGCTCAG
27FYM-A-49	ACTGACAGCCAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGACAGCCATAGAGTTTGATYMTGGCTCAG
27FYM-A-50	ACTGATCCTAGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGATCCTAGTAGAGTTTGATYMTGGCTCAG
27FYM-A-51	ACTGTACGCGTA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTACGCGTAAGAGTTTGATYMTGGCTCAG
27FYM-A-52	ACTGTCGAAGCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTCGAAGCTAGAGTTTGATYMTGGCTCAG
27FYM-A-53	ACTGTGACTTCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTGTGACTTCAAGAGTTTGATYMTGGCTCAG
27FYM-A-54	ACTTGTAGCAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGACTTGTAGCAGCAGAGTTTGATYMTGGCTCAG
27FYM-A-55	AGAACACGTCTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGAACACGTCTCAGAGTTTGATYMTGGCTCAG

Appendix A

Primer name	Golay barcode	Full primer 5'-3'
27FYM-A-56	CTAACGCAGTCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAACGCAGTCAAGAGTTTGATYMTGGCTCAG
27FYM-A-57	CTACACAAGCAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACACAAGCACAGAGTTTGATYMTGGCTCAG
27FYM-A-58	CTACATCTAAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACATCTAAGCAGAGTTTGATYMTGGCTCAG
27FYM-A-59	CTACGCGTCTCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACGCGTCTCTAGAGTTTGATYMTGGCTCAG
27FYM-A-60	CTACTACAGGTG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACTACAGGTGAGAGTTTGATYMTGGCTCAG
27FYM-A-61	CTACTGATATCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTACTGATATCGAGAGTTTGATYMTGGCTCAG
27FYM-A-62	CTAGAACGCACT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAGAACGCACTAGAGTTTGATYMTGGCTCAG
27FYM-A-63	CTAGAGACTCTT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAGAGACTCTTAGAGTTTGATYMTGGCTCAG
27FYM-A-64	CTAGCGAACATC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAGCGAACATCAGAGTTTGATYMTGGCTCAG
27FYM-A-65	CTAGTCAGCTGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAGTCAGCTGAAGAGTTTGATYMTGGCTCAG
27FYM-A-66	CTATAGTCGTGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTATAGTCGTGTAGAGTTTGATYMTGGCTCAG
27FYM-A-67	CTATCAGTGTAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTATCAGTGTACAGAGTTTGATYMTGGCTCAG
27FYM-A-68	CTATGCTTGATG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTATGCTTGATGAGAGTTTGATYMTGGCTCAG
27FYM-A-69	CTCAATGACTCA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCAATGACTCAAGAGTTTGATYMTGGCTCAG
27FYM-A-70	CTCATGTACAGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCATGTACAGTAGAGTTTGATYMTGGCTCAG
27FYM-A-71	CTCCTACTGTCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCCTACTGTCTAGAGTTTGATYMTGGCTCAG
27FYM-A-72	CTCGAGAGTACG	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGAGAGTACGAGAGTTTGATYMTGGCTCAG
27FYM-A-73	CTCGATTAGATC	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGATTAGATCAGAGTTTGATYMTGGCTCAG
27FYM-A-74	CTCGCACATATA	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCACATATAAGAGTTTGATYMTGGCTCAG

Appendix A

Primer name	Golay barcode	Full primer 5'-3'
27FYM-A-75	CTCTCTACCTGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCTCTACCTGTAGAGTTTGATYMTGGCTCAG
27FYM-A-76	TAACAGTCGCTG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAACAGTCGCTGAGAGTTTGATYMTGGCTCAG
27FYM-A-77	TAACTCTGATGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAACTCTGATGCAGAGTTTGATYMTGGCTCAG
27FYM-A-78	TAAGCGCAGCAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGCGCAGCACAGAGTTTGATYMTGGCTCAG
27FYM-A-79	TACACACATGGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACACACATGGCAGAGTTTGATYMTGGCTCAG
27FYM-A-80	TACACGATCTAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACACGATCTACAGAGTTTGATYMTGGCTCAG
27FYM-A-81	TACAGATGGCTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACAGATGGCTCAGAGTTTGATYMTGGCTCAG
27FYM-A-82	TACAGTCTCATG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACAGTCTCATGAGAGTTTGATYMTGGCTCAG
27FYM-A-83	TACATCACCACA	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACATCACCACAAGAGTTTGATYMTGGCTCAG
27FYM-A-84	TACGATGACCAC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGATGACCACAGAGTTTGATYMTGGCTCAG
27FYM-A-85	TACGGTATGTCT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGGTATGTCTAGAGTTTGATYMTGGCTCAG
27FYM-A-86	TACGTGTACGTG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACGTGTACGTGAGAGTTTGATYMTGGCTCAG
27FYM-A-87	TACTAATCTGCG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTAATCTGCGAGAGTTTGATYMTGGCTCAG
27FYM-A-88	TACTACATGGTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTACATGGTCAGAGTTTGATYMTGGCTCAG
27FYM-A-89	TACTGCGACAGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTGCGACAGTAGAGTTTGATYMTGGCTCAG
27FYM-A-90	TACTGGACGCGA	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTGGACGCGAAGAGTTTGATYMTGGCTCAG
27FYM-A-91	TACTTCGCTCGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACTTCGCTCGCAGAGTTTGATYMTGGCTCAG
27FYM-A-92	TAGACTGTACTC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGACTGTACTCAGAGTTTGATYMTGGCTCAG
27FYM-A-93	TAGAGAGAGTGG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGAGAGAGTGGAGAGTTTGATYMTGGCTCAG

Appendix A

Primer name	**Golay barcode**	**Full primer 5'-3'**
27FYM-A-94	TAGATCCTCGAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGATCCTCGATAGAGTTTGATYMTGGCTCAG
27FYM-A-95	TAGCACACCTAT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGCACACCTATAGAGTTTGATYMTGGCTCAG
27FYM-A-96	TAGCATCGTGGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGCATCGTGGTAGAGTTTGATYMTGGCTCAG
27FYM-A-97	TCCTGAGATACG	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTGAGATACGAGAGTTTGATYMTGGCTCAG
27FYM-A-98	TCGAATCACAGC	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGAATCACAGCAGAGTTTGATYMTGGCTCAG
27FYM-A-99	TCGACTCCTCGT	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGACTCCTCGTAGAGTTTGATYMTGGCTCAG
27FYM-A-100	TCGAGACGCTTA	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGAGACGCTTAAGAGTTTGATYMTGGCTCAG
519-R-B	None	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGWATTACCGCGGCKGCTG

Appendix B: Published paper

OPEN ACCESS Freely available online

PLOS ONE

Bacterial Community Development in Experimental Gingivitis

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Abstract

Current knowledge of the microbial composition of dental plaque in early gingivitis is based largely on microscopy and cultural methods, which do not provide a comprehensive description of oral microbial communities. This study used 454-pyrosequencing of the V1–V3 region of 16S rRNA genes (approximately 500 bp), and bacterial culture, to characterize the composition of plaque during the transition from periodontal health to gingivitis. A total of 20 healthy volunteers abstained from oral hygiene for two weeks, allowing plaque to accumulate and gingivitis to develop. Plaque samples were analyzed at baseline, and after one and two weeks. In addition, plaque samples from 20 chronic periodontitis patients were analyzed for cross-sectional comparison to the experimental gingivitis cohort. All of the healthy volunteers developed gingivitis after two weeks. Pyrosequencing yielded a final total of 344 267 sequences after filtering, with a mean length of 354 bases, that were clustered into an average of 299 species-level Operational Taxonomic Units (OTUs) per sample. Principal coordinates analysis (PCoA) plots revealed significant shifts in the bacterial community structure of plaque as gingivitis was induced, and community diversity increased significantly after two weeks. Changes in the relative abundance of OTUs during the transition from health to gingivitis were correlated to bleeding on probing (BoP) scores and resulted in the identification of new health- and gingivitis-associated taxa. Comparison of the healthy volunteers to the periodontitis patients also confirmed the association of a number of putative periodontal pathogens with chronic periodontitis. Taxa associated with gingivitis included *Fusobacterium nucleatum* subsp. *polymorphum*, *Lachnospiraceae* [G-2] sp. HOT100, *Lautropia* sp. HOTA94, and *Prevotella oulorum,* whilst *Rothia dentocariosa* was associated with periodontal health. Further study of these taxa is warranted and may lead to new therapeutic approaches to prevent periodontal disease.

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Competing Interests: David Bradshaw is an employee of GlaxoSmithKline. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials. The authors have declared that no other competing interests exist.

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Introduction

Gingivitis is a reversible form of periodontal disease characterized by inflammation of the gingivae in response to a mature dental plaque biofilm. In susceptible individuals persistent gingivitis may lead to chronic periodontitis [1], which causes irreversible destruction of periodontal tissue. Currently, the major means of prevention are oral hygiene practices such as tooth brushing, interdental cleaning and the use of antimicrobial mouth rinses. Many individuals do not practice oral hygiene to a standard sufficient to prevent gingivitis and alternative preventive strategies are therefore desirable. Recently, there has been interest in the potential for using probiotics or prebiotics that aim to promote periodontal health by maintaining plaque in a health-associated state [2–4]. However, a more comprehensive knowledge of the bacterial composition of plaque in health, and the changes that occur during the initial stages of gingivitis are first required. The microbiota associated with chronic periodontitis has been investigated in more depth and was recently the subject of an extensive review [5].

The essential role of plaque in gingivitis was first shown using an 'experimental gingivitis' model [6,7]. Using microscopy, the investigators noted changes in the predominant bacterial morphotypes present in plaque during the transition from health to gingivitis. In particular, they reported that early plaque in health consisted of a relatively simple bacterial community dominated by Gram-positive cocci and rods. As plaque matured, and gingivitis developed, the communities became increasingly complex with higher proportions of Gram-negative rods, fusiforms, filaments, spirilla and spirochetes. Later experimental gingivitis studies using culture confirmed these findings and provided more information regarding the specific bacterial species present in plaque [8–10]. It has been estimated, however, that approximately half of the bacteria found in the oral cavity have not been, or cannot be, cultivated in the laboratory [11]. Therefore, culture studies alone could not provide a comprehensive description of the microbiota in experimental gingivitis.

The introduction of culture-independent molecular methods to identify the bacteria present in complex samples, such as those based on cloning and Sanger sequencing of 16 S ribosomal RNA genes, has greatly expanded our knowledge of oral bacterial

communities in health and disease [12]. Aas et al. [13] used this approach to characterize the bacterial communities at nine different oral sites in five healthy individuals and detected between 34 and 72 different species-level phylotypes per individual. The authors found that particular phylotypes showed site- and subject-specificity, while others such as *Streptococcus mitis* and *Granulicatella adiacens* were detected in the majority of the subjects and sites sampled. The so-called 'red complex' putative periodontal pathogens (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*), previously associated with chronic periodontitis, and each other, on the basis of checkerboard DNA-DNA hybridization [14], were not detected. Interestingly, though, a similar later investigation of the microbiota in 10 healthy individuals detected all three 'red complex' species at low numbers [15]. No previous reported studies have used 16S rRNA gene cloning and sequencing to characterize the microbiota in experimental gingivitis.

Whilst 16S rRNA gene cloning and Sanger sequencing has undoubtedly been useful, it suffers from being relatively low-throughput and researchers have typically sequenced a limited numbers of clones per sample (often $\leq$100) [13,16]. In recent years, next-generation sequencing technologies have led to significant improvements in the depth and scale of 16S rRNA gene sequencing studies [17]. Using 454-pyrosequencing of 16S rRNA genes, Griffen [18] et al. and Abusleme et al. [19] recently compared the subgingival bacterial communities of chronic periodontitis patients to those of healthy individuals. In both cases the authors elucidated significant differences between cohorts in terms of the membership and structure of their subgingival communities and reported a higher bacterial diversity in disease. In addition, the authors revealed associations between specific bacterial taxa and disease. The 'red complex' species were associated with disease in both studies. Interestingly, however, they also identified a range of additional species that showed associations with chronic periodontitis. *Filifactor alocis* and *Treponema medium* were among those taxa most strongly associated with disease in both studies. One 454-pyrosequencing study examined bacterial community differences in the saliva and plaque of three healthy individuals and three individuals with gingivitis [20]. The communities in plaque, but not in saliva, differed significantly between health and gingivitis and a number of species-level OTUs in plaque were enriched or reduced in the individuals with gingivitis versus healthy individuals. Many of the OTUs associated with gingivitis were identified as members of the genera *Leptotrichia* and *Selenomonas*, although species-level assignments were not provided in most cases. One limitation of this and other cross-sectional studies though, is that large inter-individual variation, particularly at the species level, has been found in the oral microbiome [21–23].

This study aimed to use the longitudinal experimental gingivitis model, high-throughput 16S rRNA pyrosequencing and non-selective culture methods to comprehensively characterize the bacterial communities in plaque during the transition from health to experimentally-induced gingivitis. In addition, plaque samples from a group of patients with severe periodontitis were pyrosequenced as a cross-sectional comparison to the healthy cohort. The analyses sought to identify specific bacterial taxa associated with periodontal health and the initial stages of gingivitis.

Materials and Methods

Subject recruitment

Ethical approval for the study was granted by the South East London Research Ethics Committee 1 (formerly Guy's REC) and informed consent was obtained from all individuals who participated. All patients and subjects enrolled in the study had at least 20 teeth and were systemically healthy with no history of antibiotic use for at least three months prior to the study. Pregnant women, current smokers or individuals who quit smoking within the previous five years were not enrolled.

Periodontally healthy volunteers. Recruitment was made from clinical staff within the King's College London Dental Institute. All subjects recruited had no evidence of periodontitis and already had only minimal gingival inflammation with no need for professional intervention. The BPE (Basic Periodontal Examination) was used to screen potential subjects. Subjects had no BPE score greater than two in any of the sextants, no probing attachment loss, fewer than 15% sites bleeding after probing, and no evidence of gingival recession. All clinical examinations were undertaken by the same dentally qualified clinician.

Patients with chronic periodontitis. Patients were enrolled from those referred to the Department of Periodontology at Guy's and St Thomas' Foundation Trust for treatment. All patients had a minimum of 20 teeth and were diagnosed with severe chronic periodontitis [24]. Each patient had at least six teeth with probing depths of $\geq$6 mm and bone loss. Patients had a minimum of 20 teeth and at least six teeth with probing depths of $\geq$6 mm.

Experimental gingivitis

A summary of the experimental gingivitis study design is shown in Figure 1. Subjects were instructed to abstain from all methods of tooth cleaning in the mandible for two weeks. A soft acrylic stent was made to cover the mandibular teeth whilst brushing the maxillary teeth and was removed immediately after brushing. The soft stent could be easily inserted and removed without disturbance of the developing plaque whilst ensuring subjects could not brush their mandibular teeth and thus disrupt plaque formation. At baseline and after two weeks six sites/tooth were probed and bleeding on probing (BoP) was assessed. Probing after one week of plaque accumulation was avoided since bleeding in the crevice might have influenced the developing biofilm. However, at all time points, non-invasive samples of gingival crevicular fluid (GCF) were collected before clinical measurements or plaque sampling as the volume of GCF has been shown to increase before the development of clinically evident inflammation eg. BoP. The number of sites with clearly visible plaque (Silness and Loe plaque index = 2 [25]) were recorded. After two weeks all subjects had their teeth polished and they resumed normal oral hygiene practices.

Sample collection

Experimental gingivitis. At baseline, after collection of GCF and clinical measurements, plaque samples were collected using a sterile curette from all the mandibular teeth in the healthy subjects with the exception of the third molars. Plaque samples were collected using a sterile curette from just above the gingival margin and from the gingival crevices into 1 ml of sterile 0.1x Tris-EDTA and plaque from all sites within the patient was pooled. The same method of collection was used throughout the study to ensure that samples were of a comparable nature. GCF was collected from the mesiobuccal sites of 12 mandibular teeth on periopaper strips and the volume of fluid estimated using a Periotron 8000. Samples of plaque and GCF were collected again after one and two weeks of plaque accumulation. Plaque was collected from teeth on one half of the mandible after one week and the other half after two weeks.

Chronic periodontitis. Superficial plaque samples were collected in the same way as those from the healthy subjects. For 14 of the 20 patients, separate samples of subgingival plaque

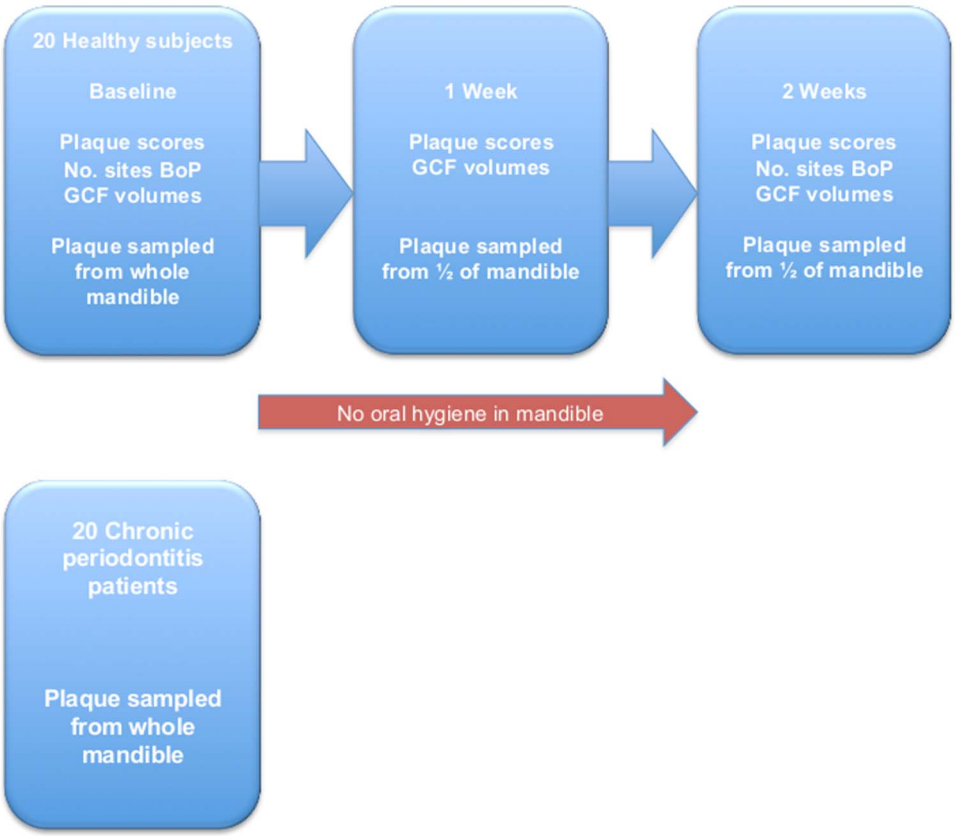

Figure 1. Study design.
doi:10.1371/journal.pone.0071227.g001

were collected by inserting a curette to the full depth of pockets >6 mm after the superficial plaque had been collected. These sites were selected on the basis of pocket depth to represent a distinctly different microbial habitat from the superficial samples and the specific sites selected varied between individuals depending on the pattern of their disease. The clinical condition of the patients was so different from the group of healthy volunteers it was impossible to blind the examiner to which group the individuals came from. However, the use of a sampling technique that removed plaque from around the gingival margins and to the depth of a healthy crevice in both the experimental gingivitis and periodontitis groups standardized the physical environment from which the superficial plaque samples were collected. The superficial plaque from the healthy volunteers and periodontitis patients was used for inter-group comparison and an additional comparison was made between the superficial and subgingival samples within the patients.

DNA extraction

DNA was extracted from the samples using the GenElute Bacterial DNA Extraction Kit (Sigma-Aldrich). Extractions were carried out following the manufacturer's instructions with an additional lysis step to increase the recovery of Gram-positive bacterial DNA: samples were incubated with a 45 mg/ml lysozyme solution at 37°C for 30 minutes. This protocol has been shown to be effective for the extraction of DNA from mock communities comprised of a mixture of Gram-positive and Gram-negative oral bacterial species (unpublished data). Extracted DNA was stored at −70°C until further processing.

16S rRNA gene PCR and 454 pyrosequencing

An approximately 500 bp region of the 16 S rRNA gene (covering V1–V3) was PCR-amplified from extracted DNA samples using composite fusion primers comprising universal 16 S primers (27FYM [26] and 519R [27]) along with Roche GS-FLX Titanium Series adapter sequences (A & B) for 454 pyrosequencing using the Lib-L emPCR method. Previously described unique 12 base error-correcting Golay barcode sequences [28] were incorporated into the forward primers (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-NNNNNNNNNNNN-AGAGTTTGATYMTGGCTCAG-3′) to enable pooling of samples in the same sequencing run. The appropriate barcoded A-27FYM and the B-519R (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-GWATTACCGCGGCKGCTG-3′) primers were used in PCRs with Extensor Hi-Fidelity PCR Mastermix (Thermo Scientific). There was an initial denaturation step of 5 mins at 95°C followed by 25 cycles of 95°C for 45 s, 53°C for 45 s, 72°C for 1 m 30 s and a final extension of 72°C for 15 mins. PCR amplicons were subsequently purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions. The size and purity of the amplicons was checked using the Agilent DNA 1000 kit and the Agilent 2100 Bioanalyzer. The amplicons were quantitated by means of a fluorometric assay using the Quant-iT Picogreen fluorescent nucleic acid stain (Invitrogen) and then pooled at equimolar concentrations (1×10^9 molecules/μl). emPCR and undirectional sequencing of the libraries was performed using the Lib-L kit and Roche 454 GS-FLX Titanium sequencer by

the Centre for Haemato-Oncology, Barts Cancer Institute, Queen Mary University of London, London, UK.

Sequence analysis

Pre-processing and analysis of sequences was carried out using the mothur analysis suite v 1.26.0 [29] based on the Schloss standardized operating procedure (SOP) [30]. Sequences were first de-noised using the AmpliconNoise algorithm [31] as implemented by mothur. After de-noising, any sequences that were less than 350 bp in length and/or had one of the following: >2 mismatches in the primer, >1 mismatch in barcode regions and homopolymers of >8 bases, were removed from the dataset. The remaining sequences were trimmed to remove the primers and barcodes and aligned to the SILVA 16S rRNA reference alignment [32]. The UChime algorithm [33] was used to identify and remove chimeric sequences. Sequences were identified by BLAST against the Human Oral Microbiome Database (HOMD) [34] at ≥98.5% sequence identity. Additionally, sequences were clustered into Operational Taxonomic Units (OTUs) at a sequence dissimilarity distance of 0.015 using an average neighbour algorithm and then classified using a Naïve Bayesian classifier with the HOMD v 10.1 reference dataset. Where necessary, possible alternatives for the species identifications were given. A previously described method was used to distinguish the commensal mitis-group streptococci from the closely related pathogen *Streptococcus pneumoniae* [35]. Good's non-parametric coverage estimator [36] was used to assess the extent of sampling of communities. Diversity of the communities was calculated using Simpson's inverse diversity index [37] and the total richness of the communities was estimated using Chao1[38] and CatchAll [39]. The Jaccard Index and the thetaYC metric [40] were used to generate distance matrices from sub-sampled sequence libraries (equal to that of the library with the fewest sequences), which were visualized as dendrograms and PCoA (Principal Coordinates Analysis) plots. Three-dimensional PCoA plots were generated in R (r-project.org) using the rgl package. The β diversity of communities was also compared based on their phylogenetic relatedness. For this, a neighbour-joining tree of the sequences constructed with Clearcut [41] was analyzed by unweighted and weighted UniFrac metrics [42] as implemented by mothur and visualized as above.

Statistical analysis

The non-parametric Friedman and Wilcoxon-signed rank tests were used to test for the significance of differences in the OTU richness and diversity of samples from the different time points of experimental gingivitis. A Bonferroni correction for multiple comparisons was applied to the alpha value for pairwise comparisons. Analysis of Molecular Variance (AMOVA) [43] was performed in mothur to determine if clustering patterns seen in the PCoA plots were statistically supported by differences in the distance matrices. A Bonferroni correction for multiple comparisons was applied to the alpha value when comparing the time points of experimental gingivitis. Parsimony [44], as implemented by mothur, was used to determine if clustering in the dendrograms was significant. Associations of OTUs with time points of experimental gingivitis and BoP scores were detected using Multivariate Association with Linear Models (MaAsLin) [45]. Linear Discriminant Analysis Effect Size (LEfSe) [46] was used to detect significant differences in the relative abundances of OTUs between healthy and chronic periodontitis cohorts. The alpha values in LEfSe were set to 0.05 and an LDA threshold of 2.0 was applied. Two-sample *t*-tests and paired *t*-tests were performed in R to determine if differences in the relative abundances of phyla between the healthy and periodontitis cohort and between time points of experimental gingivitis were statistically significant. A Bonferroni correction for multiple comparisons was applied to the alpha values. Dichotomous plaque and bleeding scores were expressed as a percentage of the number of assessed sites. Percentage plaque scores were first analyzed using the Friedman test for non-parametric data to establish whether there was a statistically significant difference in the amount of plaque over the three time points and this was followed by comparison between time points using Wilcoxon sign-rank tests. Since percentage bleeding scores were only measured at two time points they were analyzed using a Wilcoxon sign-rank test. The volume of GCF was a continuous, normally distributed variable and was therefore first analyzed using a repeated measures analysis of variance and subsequently comparison between individual time points assessed using paired t-tests.

Cultural analysis

For ten healthy subjects, baseline and two week plaque samples were cultured on non-selective media: First, 500µl of a 0.1x TE suspension containing plaque was sonicated for 20 s. Ten-fold serial dilutions of the sonicated samples (up to 10^5) were made in pre-reduced transport medium and inoculated onto Blood Agar Base no. 2+5% v/v horse blood (BA) and Fastidious Anaerobe Agar +5% v/v horse blood (FAA) in triplicate. BA plates were incubated for 4 days in 5% $C0_2$ + air at 37°C, whilst FAA plates were incubated for 10 days in an anaerobic cabinet with an atmosphere of 80% N_2, 10% H_2 and 10% $C0_2$ at 37°C. After incubation, the plates were counted and 96 colonies (48 from BA and 48 from FAA) were subcultured at random [47]. Isolates were identified by 16S rRNA gene sequence analysis [48] and analyzed using mothur as described above.

Investigation of potentially novel oral taxa

Phylotypes which did not match taxa in HOMD (<98.5% sequence identity) and were present in at least two individuals were investigated further. Specific forward primers were designed from the 454 sequences and used to amplify the 16 S rRNA gene of the target phylotype in combination with the primer 1492R. PCR amplicons of the correct size were subsequently cloned into *Escherichia. coli* using the TOPO cloning kit (Invitrogen, UK) and sequenced as described previously [48].

Results

Clinical results

The experimental gingivitis cohort comprised 16 females and four males with a mean age of 28.1 (± 2.1). 19 of the subjects completed the study. One subject withdrew from the study because of a chest infection. No other adverse affects or complications were reported. After two weeks all subjects had developed thick clearly visible plaque on most tooth surfaces. They had significantly increased gingival bleeding on probing (BoP) scores (Table 1) and the mean volume of gingival crevicular fluid (GCF) increased significantly after one and two weeks. A total of 11 female and nine male chronic periodontitis patients with a mean age of 48.5 (± 9) were sampled. A two-sample *t*-test showed that periodontitis patients were significantly older than the experimental gingivitis group. The mean number of teeth per patient was 28.1 (±2.8) whilst the number of teeth per patient with pocket depths ≥6 mm was 11.9 (±5.9). In 14 of the patients, subgingival plaque samples from deep pockets were obtained as an additional comparison to the plaque samples from around the gingival margin.

Table 1. Summary of clinical parameters of subjects during experimental gingivitis.

Time point	GCF volume (μl)	% of sites with Bleeding on Probing (BoP).	% of sites with visible plaque.
	Mean (±SD)	Median (IQR)	Median (IQR)
Baseline	6.82 (±2.01)	7.04 (4.8–10.7)	6.7 (1.8–10.0)
1 Week	8.22 (±1.94)	N/A	62.4 (50.1–79.5)
2 Weeks	9.77(±2.21)	37.2 (29.8–47.2)	87.02 (71.1–92.9)

GCF volume increased over 2 weeks ($P<0.0001$) and increases were significant at 1 and 2 weeks ($P<0.019$). The % of sites bleeding increased over 2 weeks ($p<0.0001$). The % of sites with visible plaque increased over 2 weeks ($P< 0.0001$) and increases were significant at 1 and 2 weeks ($P<0.0001$).
doi:10.1371/journal.pone.0071227.t001

Pyrosequencing summary

394 558 sequences with a mean length of 423 bases were obtained after initial quality filtering. Alignment to the SILVA reference database, subsequent screening of the alignment and removal of chimeras resulted in a final dataset of 344 267 high quality sequences (of which 31 604 were unique) with a mean length of 354 bases. This provided a final mean yield of 3742 (±789) sequences per sample for further analysis.

OTU-based alpha and beta diversity of bacterial communities in plaque

Clustering of sequences into OTUs at a distance of 0.015 resulted in 89 to 394 (median 299) species-level OTUs per plaque sample/bacterial community. Mean coverage of the communities according to Good's non-parametric was 96.9% (±0.7%). Chao1 estimates of total OTU richness ranged between 130 and 634 OTUs (median 464), whilst CatchAll gave estimates from 200 to 1690 (median 644). The observed OTU richness and diversity of communities (Simpson's inverse diversity index) at different sampling times of experimental gingivitis are shown in Figure 2. The richness of the communities across time points was significantly different (Friedman test, $P<0.016$). Using pairwise Wilcoxon signed-rank tests there was no significant difference between baseline communities and the one- and two-week communities. However, the number of OTUs was significantly higher in two-week communities compared to one-week communities ($P<0.01$). There was a significant difference in diversity across time points (Friedman test, $P<0.00044$). Pairwise Wilcoxon-signed rank tests showed that diversity was significantly higher in two-week communities compared to both baseline ($P<0.0001$) and one-week communities ($P<0.0012$), but that there was no significant difference between baseline and one-week communities. A table summarizing the alpha diversity parameters for each of the 92 samples analyzed is shown in the supporting information (Table S1). Only eight OTUs, assigned to the taxa: *Actinobaculum* sp. HOT183, *Campylobacter gracilis*, *Campylobacter showae*, *Cardiobacterium hominis*, *Fusobacterium nucleatum* subsp. *polymorphum*, *Lautropia mirabilis*, *Streptococcus mitis*/ HOT064/ HOT423/ HOTA95/ HOTE14, and *Veillonella parvula*, were detected in all healthy subjects' baseline samples. Three OTUs, assigned to the taxa *Fusobacterium nucleatum* subsp. *animalis*, *Fusobacterium nucleatum* subsp. *vincentii*, and *Streptococcus mitis*/ HOT064/ HOT423/ HOTA95/ HOTE14 were shared among all of the periodontitis patients.

Comparisons of the bacterial community membership (Jaccard index and unweighted UniFrac) of plaque samples using dendrograms, showed that communities from the different time points of experimental gingivitis clustered principally by subject (Figure S1). A separate comparison of baseline plaque communities from the healthy cohort to superficial plaque from chronic periodontitis patients showed some clustering by cohort (Figure S2). Interestingly, a separate analysis of superficial and subgingival plaque samples from periodontitis patients showed that communities clustered by patient rather than by the type of plaque sample (Figure S3). A parsimony analysis was conducted for each dendrogram to assess the significance of clustering patterns of groups and time points. There was a significant difference between the clustering of the baseline communities of healthy subjects and superficial plaque communities of periodontitis patients ($P<0.036$ and $P<0.012$ for Jaccard index and unweighted UniFrac dendrograms, respectively). There were no significant differences between the clustering of communities from different time points of experimental gingivitis or between superficial and subgingival plaque communities from periodontitis patients.

Comparison of the bacterial community structure of plaque samples was performed using the thetaYC and weighted UniFrac metrics from which distance matrices were constructed and visualized using PCoA (Figures 3 and 4). For the experimental gingivitis cohort, both plots showed spatial separation of one- and two-week communities from baseline communities (Figure 3). In addition, separate PCoA analyses comparing superficial periodontitis communities with healthy baseline communities (Figure 4) and two-week gingivitis (not shown) communities showed separation in each case. AMOVA tests found an overall significant difference between the three time points of experimental gingivitis for both the thetaYC and weighted UniFrac distances ($P<0.0016$ and $P<0.001$, respectively). Pairwise AMOVA comparisons showed that there were significant differences between baseline and one-week communities ($P<0.017$ and $P<0.001$ for thetaYC and weighted UniFrac, respectively) and between baseline and two-week communities ($P<0.0006$ and $P<0.001$ for thetaYC and weighted UniFrac, respectively). However, there was no significant difference between one- and two-week communities ($P = 0.526$ and $P = 0.098$ for thetaYC and weighted UniFrac respectively). AMOVA tests also confirmed that there were significant differences between the baseline communities of healthy subjects and the superficial communities of the periodontitis patients ($P = 0.0095$ and $P<0.001$ for thetaYC and weighted UniFrac, respectively). Similarly, there was a significant difference between the two-week communities and the superficial periodontitis samples ($P<0.0005$ for both thetaYC and weighted UniFrac). There was no significant difference in the thetaYC or weighted UniFrac distances between the superficial and subgingival periodontitis samples from periodontitis patients by AMOVA testing.

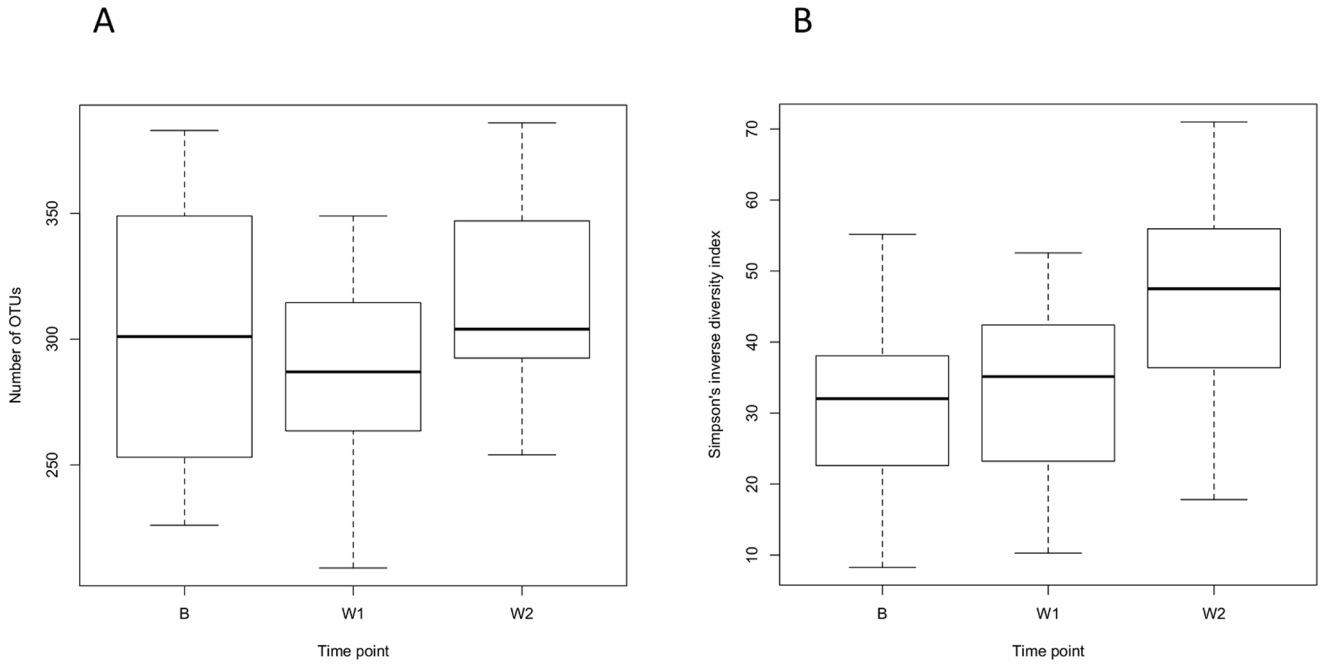

Figure 2. Richness and diversity of plaque during the induction of experimental gingivitis. Box-and-whisker plots comparing the species-level OTU richness and diversity of time points during experimental gingivitis. The top and bottom boundaries of the boxes show the 75th and 25th percentile and the ends of the whiskers show the maximum and minimum values. Bold lines within the boxes represent median values (50th percentile). (**A**) Number of observed OTUs at baseline (B), one week (W1) and two weeks (W2). (**B**) Simpson's inverse diversity index at baseline (B), one week (W1) and two weeks (W2).
doi:10.1371/journal.pone.0071227.g002

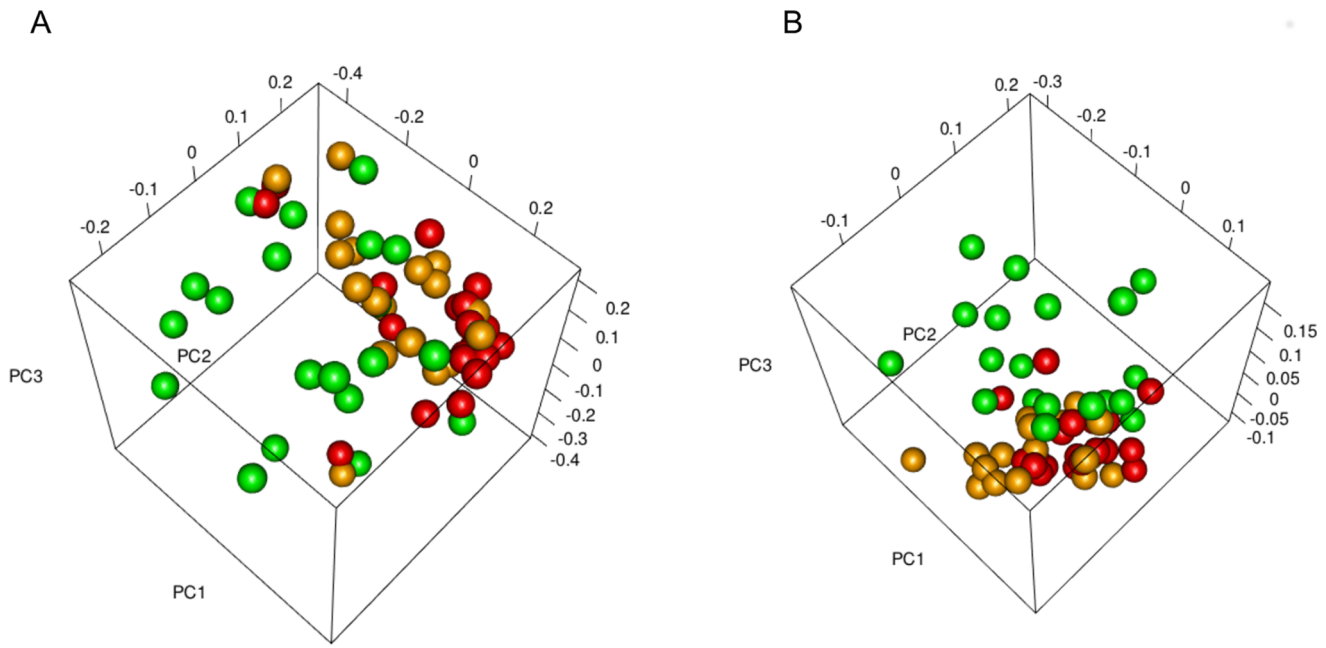

Figure 3. Shifts in bacterial community structure of plaque during the induction of experimental gingivitis. PCoA plots comparing community structure of plaque samples from different time points of experimental gingivitis. Baseline samples are colored green, one-week samples are orange and two- week samples are red. (**A**) PCoA based on the thetaYC calculator. PC1 = 12.11% of variance explained, PC2 = 8.89%, PC3 = 5.55% (**B**) PCoA based on the weighted UniFrac calculator. PC1 = 19.78% of variance explained, PC2 = 10.65%, PC3 = 5.65%.
doi:10.1371/journal.pone.0071227.g003

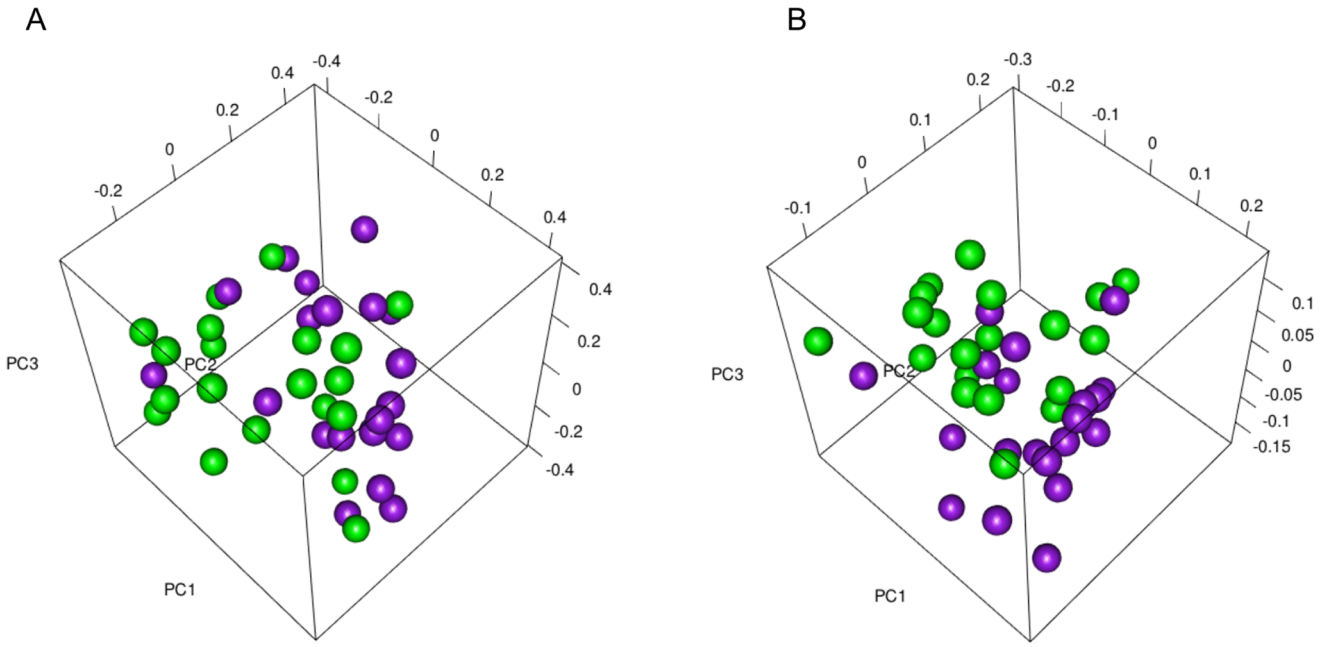

Figure 4. Bacterial community structure of plaque in health and chronic periodontitis. PCoA plots comparing community structure of baseline plaque samples from the healthy cohort to supragingival plaque samples of chronic periodontitis patients. Plaque communities from healthy individuals are colored green and those from periodontitis are colored purple. (**A**) PCoA based on the thetaYC calculator, PC1 = 16.38% of variance explained, PC2 = 10.09%, PC3 = 8.02% (**B**) PCoA based on the weighted UniFrac calculator, PC1 = 22.12% of variance explained, PC2 = 8.89%, PC3 = 6.85%.
doi:10.1371/journal.pone.0071227.g004

OTU-level composition and shifts of bacterial communities

The 10 OTUs detected with the highest relative abundance across all 92 samples were assigned to the taxa *Streptococcus sanguinis*, *Rothia dentocariosa*, *Veillonella parvula*, *Fusobacterium nucleatum* subsp. *polymorphum*, *Streptococcus mitis*/ HOT064/ HOT423/ HOTA95/ HOTE14, *Streptococcus cristatus*/ HOT071, *Fusobacterium nucleatum* subsp. *vincentii*, *Lautropia mirabilis*, *Porphyromonas gingivalis* and *Leptotrichia buccalis*. The intra-sample relative abundances of the top 50 OTUs detected are shown in the supporting information (Dataset S1). Statistical analysis using linear models implemented in MaAsLin was initially performed to determine correlations of OTUs with sampling times during experimental gingivitis. A full list of the resulting OTUs that were positively or negatively correlated with one- and/or two-week time points of experimental gingivitis are shown in Table S2. Box plots showing changes in relative abundance over time for the most significant OTUs are shown in Figure S4. A second analysis in MaAsLin included bleeding on probing (BoP) scores and the baseline and two-week time points. OTUs were statistically associated with the clinical condition indicated by BoP scores, rather than the time point at which the samples were collected. The OTUs identified as *Lautropia* sp. HOTA94, *Lachnospiraceae* sp. HOT100, *Prevotella oulorum* and *Fusobacterium nucleatum* subsp. *polymorphum* were most significantly positively correlated with BoP (P and Q values <0.05) whilst an OTU identified as *Rothia dentocariosa* was most significantly negatively correlated with BoP. A full list of OTUs that were correlated with BoP scores is shown in Table S3. Linear discriminant analysis using LEfSe was used to detect OTUs that had significantly different relative abundances between chronic periodontitis (superficial plaque) and health (baseline plaque). A total of 41 OTUs were found to be significantly differentially abundant between these groups (Figure 5). An OTU identified as *Porphyromonas gingivalis* was most strongly associated with chronic periodontitis.

BLAST identification using the HOMD reference set

BLAST summary. Following BLAST, 331 398 of the 344 267 sequences (96.3%) were mapped to taxa in the HOMD reference set. The 331 398 sequences were assigned to a total of 11 phyla, 126 genera and 567 species-level phylotypes/groups. 3.7% of the 344 267 sequences were not assigned (<98.5%) to any known phylotypes in the HOMD reference set.

Phylum level composition. The predominant phyla detected across all plaque samples in order of mean relative abundance in samples were *Firmicutes* (31.3%), *Fusobacteria* (19.0%), *Bacteroidetes* (18.9%), *Actinobacteria* (14.0%), and *Proteobacteria* (13.9%). Other phyla detected, but which were not present in every sample, included TM7, *Synergistetes*, *Spirochaetes*, SR1, *Chloroflexi* and GN02. There was considerable inter-individual variability in the relative abundance of phyla, both among healthy individuals and patients with chronic periodontitis. However, comparison of the mean relative abundances of the predominant phyla for each cohort (Figure S5) showed that *Proteobacteria* were significantly more abundant in health (two sample *t*-test: $P<0.0056$) whilst *Bacteroidetes* were significantly more abundant in periodontitis ($P<0.0039$). Furthermore, the phyla *Synergistetes* and *Spirochaetes* were detected in 90% and 95% of the periodontitis patients and 40% and 80% of the healthy subjects, respectively. The phylum *Chloroflexi* was not detected in health but found in 15% of the periodontitis patients at low levels. Phylum-level shifts within and across all subjects were observed during experimental gingivitis (Figure S6). Specifically, the relative abundance of *Actinobacteria* was significantly higher at baseline compared to one and two weeks (paired *t*-test: $P<0.001$ for both comparisons), whilst the *Bacteroidetes* were significantly higher in one- and two-week samples

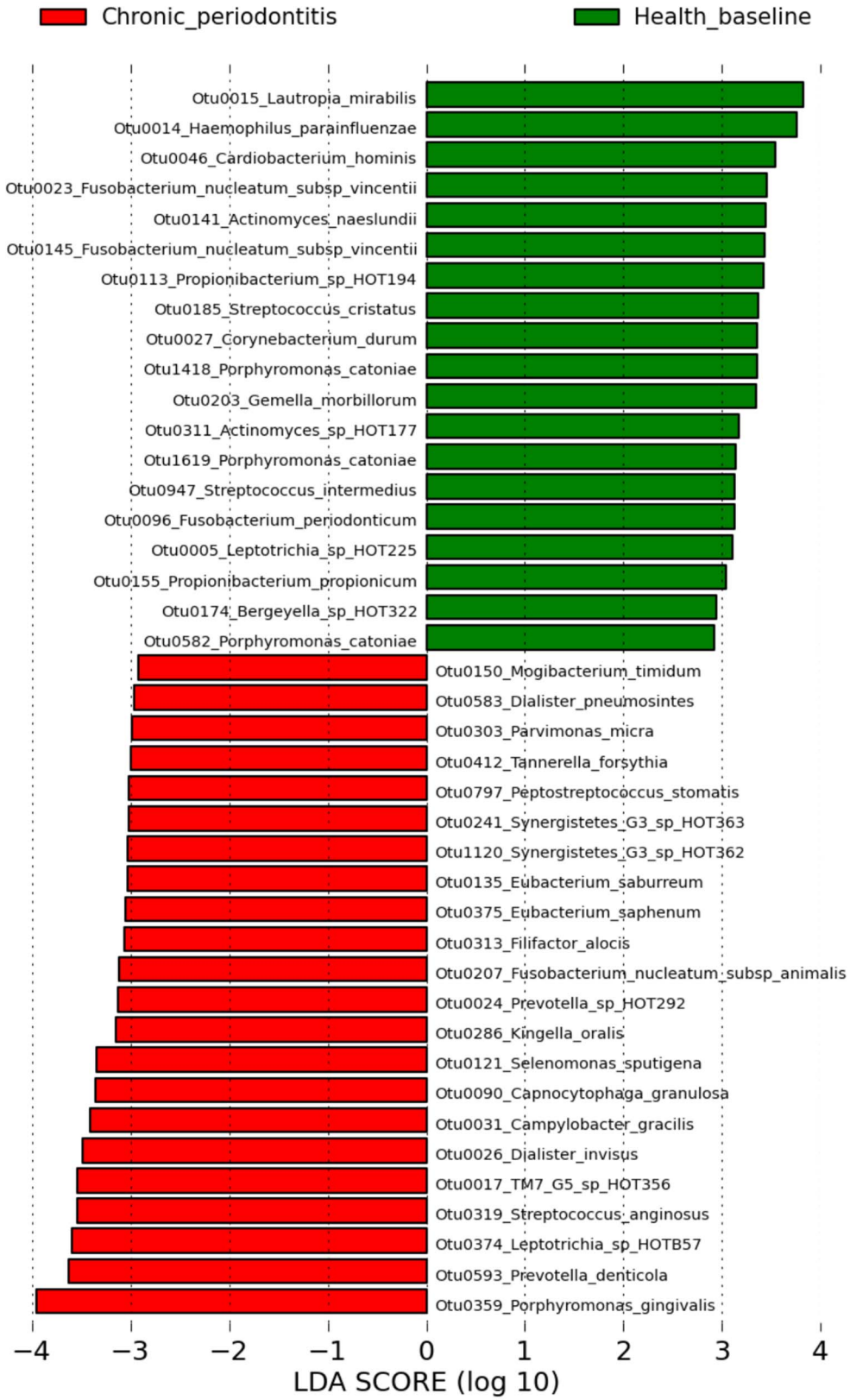

Figure 5. Detection of differentially abundant OTUs in health and chronic periodontitis. Differentially abundant OTUs between baseline plaque communities of the experimental gingivitis cohort and superficial plaque communities in chronic periodontitis patients as identified by LEfSe. OTUs are ranked by their LDA effect size. OTUs associated with healthy subjects are shown in green and OTUs associated with chronic periodontitis are shown in red.
doi:10.1371/journal.pone.0071227.g005

compared to baseline ($P<0.001$ for both comparisons). The shifts in phyla during experimental gingivitis, however, showed considerable variability among individuals. For example, subject four showed a striking drop in their relative abundance of *Actinobacteria*, from 41.6% at baseline to 9.3% after two weeks. In contrast, the relative abundances of *Actinobacteria* in subject five were 15.1% at baseline and 18.5% after two weeks.

Culture-based community analysis

Baseline and two week plaque samples were cultured for 11 of the healthy subjects. For one subject, only the baseline sample was obtained (subject two). 1935 of 1956 isolates were assigned to taxa in the HOMD extended reference set V1.1 using BLAST (≥98.5% sequence identity). These sequences represented five phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria*) and 43 genera. The phylotypes/ groups detected with the highest relative abundance using both incubations (anaerobic and air +5% $C0_2$) were *Streptococcus sanguinis* and the *Actinomyces naeslundii* group. *S. sanguinis* was the most abundant isolate at baseline with a median relative abundance of 18.8% and 13.8% using air +5% $C0_2$ and anaerobic incubations respectively. After two weeks the *A. naeslundii* group was the most abundant (21.7% and 14.0% using air +5% $C0_2$ and anaerobic incubations respectively). The observed OTU richness and diversity of baseline and two-week communities by culture is summarized in Table 2.

A number of taxa isolated in culture were not detected by pyrosequencing among the same samples, including *Actinomyces* sp. HOT172, *Capnocytophaga* sp. HOT380, *Staphylococcus epidermidis* group, *Staphylococcus hominis*, *Staphylococcus warneri*, *Paenibacillus* HOTA06, and *Propionibacterium acnes*/HOT193. No representatives of the phyla GN02, *Spirochaetes*, SR1, *Synergistetes*, or TM7, were detected by culture.

Novel oral taxa

12 869 sequences in the pyrosequencing dataset were not mapped to any taxa in HOMD (<98.5% sequence similarity). These sequences represented lineages within a number of different phyla and many were found in multiple samples in both the experimental gingivitis and chronic periodontitis cohorts. Seven of these phylotypes/groups were investigated further. Virtually full length sequences were obtained for all of these phylotypes/groups using specific 16 S rRNA primers for targeted PCR, as well as for two novel cultured isolates and made available on GenBank and submitted to HOMD. A summary of these taxa, their closest known phylogenetic relatives, and Genbank accession numbers are shown in Table 3. Of particular interest was a deep branching lineage within the class *Mollicutes* representing a new order. The prevalence of these taxa in the experimental gingivitis and chronic periodontitis cohorts is shown in Table S4 and phylogenetic trees for each taxon are shown in Figures S7–15.

Discussion

This is the first study to use 454-pyrosequencing to examine the bacterial composition of dental plaque in experimental gingivitis, and one of few reported longitudinal investigations of the oral microbiome. The results of this study have shown that, in the absence of oral hygiene, the transition from periodontal health to gingivitis is accompanied by a shift in the bacterial community structure of plaque and an increase in bacterial community diversity. In addition, the results demonstrated significant differences in both the membership and structure of analogous health- and chronic periodontitis-associated plaque samples, and confirmed the association of particular species previously associated with chronic periodontitis [18,19].

A number of previous high-throughput 16S rRNA sequencing studies characterized oral bacterial communities to the phylum or genus level only [49–51]. It is important to distinguish taxa at the species-level, as different species within the same phylum and/or genus may be health-associated or pathogenic/disease-associated. The targeting of a highly variable region of the 16S rRNA gene (V1–V3) and the use of a curated human oral 16S rRNA gene reference set (HOMD), enabled the identification of OTUs (clustered at a distance of 0.015) to species-level where possible. Whilst some studies [18–20] have also recently reported species-level 16S rRNA gene pyrosequencing analysis of the bacterial communities in periodontal health, gingivitis and chronic periodontitis, these studies were cross-sectional in nature and did not examine changes in the same individuals during the transition from health to disease. In the present study a highly species-rich bacterial community (201–383 OTUs per sample) was revealed in early health-associated plaque. This richness is considerably higher than indicated by the culture data of this study (Table 2) and in previous studies characterizing the oral microbiome in health. Aas et al. [13] found between 12 and 27 species-level phylotypes on tooth surfaces and between four and 21 in subgingival plaque, while Bik et al. [15] detected between 65 and 128 species level OTUs in pooled samples from different oral surfaces. The number of species-level OTUs per plaque sample observed in health in the present study is in a similar range to other recent 16S rRNA pyrosequencing studies. Zaura et al. [52] found on average 266 species-level phylotypes (97% sequence similarity) per sample and Griffen et al. [18] detected between 100 and 300 phylotypes (98% sequence similarity) per individual. However, Huang et al. [20] reported the presence of 379–684 species-level OTUs (97% sequence similarity) in the supra-gingival plaque of healthy individuals. Comparison of the numbers of observed OTUs, or phylotypes, between this and other studies, though, is complicated

Table 2. Alpha diversity of plaque samples as analyzed by culture.

Time point (incubation)	No. of observed OTUs	Simpson's inverse diversity index	Chao 1 total OTU richness estimate
	Median (IQR)	Median (IQR)	Median (IQR)
Baseline (air +5% $C0_2$)	14.5 (11.0–21.0)	10.9 (6.6–17.9)	19.1 (15.4–36.0)
2 Weeks (air +5% $C0_2$)	18.5 (15.3–22.0)	14.6 (9.3–19.5)	30.4 (23.2–43.2)
Baseline (Anaerobic)	22.5 (18.3–24.8)	16.8 (12.7–29.2)	51.3 (31.0–71.2)
2 Weeks (Anaerobic)	24.5 (21.5–28.3)	25.0 (15.8–34.6)	57.9 (38.0–70.4)

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Table 3. Oral taxa targeted for full-length 16S rRNA gene sequencing.

Closest phylogenetic relative in HOMD (% sequence identity)	Name and accession no. of closest match in Genbank (% sequence identity)	Clone/ Isolate	Genbank accession no.	HOMD oral taxon no.
Bacillus sp. HOT A03 (84%)	Uncultured bacterium clone 4_11, HE681226 (99%)	Clone	KC203059	HOT 906
Microlunatus sp. HOT C95 (93.5%)	Uncultured *Propionibacteriaceae* bacterium clone 08_3_G04, GU227180 (99%)	Clone	KC203064	HOT 915
Prevotella sp. HOT 473 (94.3%)	Uncultured *Prevotellaceae* bacterium clone 601F05, AM420222 (99%)	Clone	KC203063	HOT 914
Actinomyces sp. HOT 449 (94.8%)	Uncultured bacterium clone 070050_018, JQ466816 (99%)	Clone	KC203057	HOT 897
Bergeyella sp. HOT 322 (96.3%)	Uncultured bacterium clone rRNA004, AY958777 (99%)	Clone	KC203058	HOT 900
Tannerella sp. HOT 808 (97.9%)	Uncultured *Tannerella* sp. clone 402C09, AM420141 (99%)	Clone	KC203065	HOT 916
Leptotrichia hofstadii (97.9%)	Uncultured *Leptotrichia* sp. clone 303F08, AM420110 (99%)	Clone	KC203062	HOT 909
Aggregatibacter sp. HOT 513 (97.9%)	Uncultured bacterium clone P1D1-738, EF511870 (98%)	Isolate	KC203060	HOT 898
Capnocytophaga sp. HOT 336 (98.3%)	*Capnocytophaga* sp. P2 oral strain P4_P12, AY429469 (98%)	Isolate	KC203061	HOT 903

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by differing methodologies such as, but not limited to, the use of different regions of the 16S rRNA gene and different sequence similarity cut-offs for OTU clustering or BLAST classification. A recent study [22] analyzed a mock bacterial community using pyrosequencing and showed that despite de-noising and stringent quality filtering, additional erroneous OTUs were detected, indicating that pyrosequencing may still over-estimate the number of OTUs present.

The species-level OTU richness detected by pyrosequencing was not significantly higher in subjects after one and two weeks without oral hygiene. This was perhaps surprising given findings to the contrary in a previous culture-based experimental gingivitis study [9]. These observations are, however, similar to that of Huang et al. [20] who, using pyrosequencing, did not note any significant difference in richness between plaque samples from healthy individuals and those with gingivitis. The significant increase in community diversity (Simpson's inverse diversity index), after two weeks of experimental gingivitis, in the absence of significantly increased richness, indicated that the increased diversity was mainly a result of increasing evenness. This suggests that as plaque accumulated, species that dominated the early communities in health decreased in relative abundance over time whilst previously minor constituent species increased in relative abundance, resulting in a more even distribution of species after two weeks of plaque accumulation. Significant differences between the bacterial community structures (using both OTU- and phylogenetic-based analyses) of plaque in health (baseline) and gingivitis (one- and two-week plaque samples) were shown by the pyrosequencing data. However, clustering comparisons on the basis of community membership indicated that inter-individual differences were greater. This was supported by the low number of OTUs that were shared between all 20 healthy subjects, suggesting a small core/shared oral microbiota in plaque at this taxonomic level.

The PCoA plots revealed a shift in bacterial community structure as gingivitis developed in the subjects following the withdrawal of oral hygiene. It is interesting to note, however, that the PCoA plots also indicated considerable variability in community structure among the healthy subjects. This variability was evident both at the phylum- and species-level. For example, the relative abundance of *Actinobacteria* ranged between 8.4% and 55.9% in baseline samples. At the species-level, OTUs that were dominant in some individuals' baseline samples were detected only at low relative abundances, or not at all, in others. An OTU identified as *Neisseria flavescens/subflava* was the dominant OTU at baseline in subject 16 (8.81% of the sequences) but was not detected in 11 of the healthy subjects. It would be useful if future studies examining the microbial composition of plaque in health and periodontal disease include a greater number of subjects, as the relatively low number (20 healthy subjects and 20 chronic periodontitis patients) in the present study was a limitation given the observed inter-individual variability. Continuing advances in high-throughput sequencing technology may facilitate this. Despite this inter-individual variability, the present study identified a number of OTUs that showed significant changes in relative abundance after one and two weeks of experimental gingivitis. The analyses also identified OTUs that were negatively or positively correlated with bleeding on probing (BoP) scores. OTUs that decreased in relative abundance over time and that were negatively correlated with BoP, were predominantly aerobic and facultatively anaerobic Gram-positive cocci and rods, including members of the genera *Actinomyces*, *Rothia*, and *Streptococcus*. It has been previously shown that *Streptococcus* spp., *Actinomyces* spp., and *Rothia* spp. are among the earliest colonizers of the tooth surface [21,53] and are prevalent in the mouths of healthy individuals [13,15] so it was unsurprising that members of these genera were abundant in health. An OTU identified as *Rothia dentocariosa* showed the most significant negative correlation with one- and two-week time points of experimental gingivitis, and with increased BoP scores. *R. dentocariosa* has previously been identified as a common constituent of the oral microbiome in health, particularly on tooth surfaces [13,15,50] and the genus *Rothia* has been associated with oral health. [18,54]. Furthermore, a recent pyrosequencing study found that *R. dentocariosa* dominated the health-associated subgingival plaque communities analyzed [19].

The OTUs that increased in relative abundance as gingivitis developed and that were positively correlated with BoP scores were mostly Gram-negative taxa of the genera *Campylobacter*, *Fusobacterium*, *Lautropia*, *Leptotrichia*, *Porphyromonas*, *Selenomonas*, and *Tannerella*. Among those that have been previously cultivated, many were obligate anaerobes. These findings are largely in accordance with the observations of Theilade et al. [7], who, in an early experimental gingivitis study, reported an increase in the proportion of Gram-negative cocci and rods as well as filaments, spirilla and spirochetes as gingivitis developed. The OTUs most strongly positively correlated with increased BoP scores included the unnamed phylotypes *Lachnospiraceae* [G-2] sp. HOT100 and *Lautropia* sp. HOTA94, as well as the named previously cultured organisms *Fusobacterium nucleatum* subsp. *polymorphum* and *Prevotella oulorum*. An interesting observation of this study was the increased relative abundance in one- and two-week time points, and positive correlation with BoP, of an OTU identified as *Tannerella* sp. HOT286 (also known as oral clone BU063). This phylotype, a close relative of the putative periodontal pathogen *Tannerella forsythia*, was previously associated with periodontal health in a study using PCR to compare its prevalence in the 25% of a population with the most severe periodontitis to its prevalence in the 25% of the population with the best periodontal health [55]. In that study, the relatively healthy subjects included some with pocket depths and attachment loss up to a maximum of 5 mm and, unlike the present study, the extent of gingival inflammation was not reported. [55]. Interestingly, *Tannerella* sp. oral clone BU063 was found to be more prevalent among individuals with gingivitis and necrotizing ulcerative gingivitis than in those with periodontitis in another study using fluorescent *in situ* hybridization [56]. Furthermore, Huang et al. [20] recently reported a significantly higher relative abundance of *Tannerella* sp. BU063 in the plaque of individuals with gingivitis than in healthy individuals.

The majority of OTUs that had a significantly higher relative abundance in chronic periodontitis patients than in healthy subjects were taxa that have been previously associated with periodontitis. However, one strongly associated OTU (*Leptotrichia* sp. HOTB57) may represent an additional taxon to add to this expanding list. *Porphyromonas gingivalis* had the greatest effect size among the associated OTUs and has been previously associated with periodontitis on the basis of both culture-dependent and independent studies [14,16,57]. Interestingly, *Prevotella denticola* was among the other strongly periodontitis-associated OTUs. *P. denticola* was previously found to increase in incidence with increasing severity of periodontal disease based on culture [58], was detected at higher prevalence in periodontitis patients than healthy subjects using species-specific PCR [16] and was strongly associated with chronic periodontitis in another recent pyrosequencing study [18]

The volunteer group of healthy subjects were all clinical staff within the Dental Institute who were highly motivated and well informed in the practise of effective oral hygiene. The clinical condition in severe chronic periodontitis contributed a substantially different clinical environment for comparison, but a convenience sample of the 20 patients with severe chronic periodontitis can not be said to represent the entire population with periodontitis. The patients with periodontitis were significantly older than the healthy volunteers and this is a limitation of the current study. For ethical reasons it would not be appropriate to monitor changes in the microbiota whilst allowing irreversible destructive disease to progress over a number of years without intervention. The effects of patient age on the microbiota can not be easily separated from the effect of the different microbial habitat which develops as the patient ages and disease progresses. This study could not have been ethically designed to monitor the microbiota as gingivitis progressed to periodontitis or as periodontitis increased in severity with age. More extensive studies would be required to compare different types of periodontal disease, different levels of disease in different age groups and populations before a truly comprehensive description of the periodontal microbiome could be described with complete confidence. However, within these limitations, the current work has successfully applied deep sequencing technology to monitor short term changes in the microbiota during the induction of reversible mild periodontal disease and contrasted it with the microbiota of a group of patients with severe and irreversible periodontal disease.

In conclusion, this study has shown the presence of a highly rich bacterial biota in health-associated plaque and determined longitudinal shifts in bacterial community structure as plaque accumulates and gingivitis develops. The analyses both identified new health- and gingivitis-associated taxa and confirmed the association of a number of putative periodontal pathogens with chronic periodontitis. Further investigation of these taxa may lead to the development of novel therapies aiming to prevent the early stages of periodontal disease.

Supporting Information

Figure S1 Clustering of plaque communities in experimental gingivitis. Dendrogram of plaque samples from all time points of experimental gingivitis compared based on their community membership using the Jaccard index. HS = healthy subject, B = baseline, 1W = one week, 2W = two weeks. Numbers indicate subject number.
(PDF)

Figure S2 Clustering of plaque communities in health and chronic periodontitis. Dendrogram of plaque samples from the baseline time point of the experimental gingivitis cohort and superficial plaque samples from patients with periodontitis, compared based on their community membership using the Jaccard index. HS = healthy subject, B = baseline, CP = chronic periodontitis patients. Numbers indicate subject or patient number.
(PDF)

Figure S3 Clustering of superficial and subgingival plaque communities in chronic periodontitis. Dendrogram of superficial and subgingival plaque samples from patients with chronic periodontitis, compared based on their community membership using the Jaccard index. CP = chronic periodontitis patients, Sub = subgingival plaque. Numbers indicate patient number.
(PDF)

Figure S4 Changes in relative abundance of OTUs during the induction of experimental gingivitis. Box plots showing significant changes in relative abundance of OTUs during induction of experimental gingivitis. Time 1 = baseline, Time 2 = 1 week, Time 3 = 2 weeks. All OTUs shown are $P<0.05$, $Q<0.05$. Correlation coefficients are shown in parentheses.
(TIF)

Figure S5 Relative abundances of the predominant phyla in health and chronic periodontitis. Histogram comparing the mean relative abundances of the predominant phyla detected in healthy subjects (baseline) and chronic periodontitis patients (superficial plaque). Statistically significant differences as indicated by two-sample t-tests are highlighted with an * and error bars shown are the standard error of the mean (SEM).
(TIF)

Figure S6 Relative abundances of the predominant phyla during the induction of experimental gingivitis.

Histogram chart comparing the mean relative abundances of the predominant phyla at the different time points of experimental gingivitis. Statistically significant differences as indicated by two-sample t-tests are highlighted with an * and error bars shown are the standard error of the mean (SEM).
(TIF)

Figure S7 Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Mollicutes*_CP6_C1, members of the *Firmicutes* phylum and other phyla found in the oral cavity. The tree was constructed using the neighbor-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.
(PDF)

Figure S8 Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Propionibacteriaceae* HS10_B_C3 and members of the phylum *Actinobacteria*. The tree was constructed using the neighbor-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.
(PDF)

Figure S9 Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between, *Alloprevotella*_HS3_1W_C6 and members of the genera *Alloprevotella* and *Prevotella*. The tree was constructed using the neighbor-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.
(PDF)

Figure S10 Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between, *Actinomyces*_HS14_2W_C6 and members of the genus *Actinomyces*. The tree was constructed using the neighbor-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.
(PDF)

Figure S11 Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Bergeyella*_HS1_1W_C3, members of the genus *Bergeyella* and other members of the class *Flavobacteria* in the phylum *Bacteroidetetes*. The tree was constructed using the neighbor-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.
(PDF)

Figure S12 Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Tannerella* CP6_C2 and members of the genera *Tannerella* and *Porphyromonas*. The tree was constructed using the neighbor-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.
(PDF)

Figure S13 Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Leptotrichia* CP4_C6 and members of the genus *Leptotrichia*. The tree was constructed using the neighbor-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.
(PDF)

Figure S14 Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Aggregatibacter*_HS19_2W_I12 and members of the genera *Aggregatibacter* and *Haemophilus*. The tree was constructed using the neighbor-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.
(PDF)

Figure S15 Phylogenetic tree based on 16S rRNA gene comparisons showing the relationship between *Capnocytophaga*_HS5_2W_I24 and members of the genus *Capnocytophaga*. The tree was constructed using the neighbor-joining method from a distance matrix constructed from aligned sequences using the Jukes-Cantor correction. Numbers represent bootstrap values for each branch based on data from 500 trees. Scale bars show the number of nucleotide substitutions per site.
(PDF)

Table S1 Alpha diversity of plaque samples.
(DOC)

Table S2 OTUs associated with time points of experimental gingivitis. OTUs were associated with time points of experimental gingivitis using Multivariate Association with Linear Models (MaAsLin). OTUs are ranked according to their *P* value. OTUs listed have *P* values <0.05.
(DOC)

Table S3 OTUs associated with bleeding on probing scores. OTUs were associated with BoP using Multivariate Association with Linear Models (MaAsLin). OTUs are ranked according to their *P* value. OTUs listed have *P* values <0.05.
(DOC)

Table S4 Prevalence of novel taxa in the experimental gingivitis and chronic periodontitis cohorts.
(DOC)

Dataset S1 Relative abundances of the top 50 OTUs in plaque samples. HS = Healthy subject, 1W = 1 week, 2W = 2 weeks, CP = Chronic periodontitis patient, Sub = subgingival plaque, Numbers indicate subject or patient number. Values given are percentage of total sequences.
(XLS)

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Author Contributions

Conceived and designed the experiments: JK VB DB WW. Performed the experiments: JK VB. Analyzed the data: JK VB WW. Wrote the paper: JK VB WW.

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